METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF STERO ISOMERIC PURITY OF ROCURONIUM BROMIDE BULK DRUG BY HPLC

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***Abstract*: A simple, sensitive and precise RP-HPLC method for the determination stereo isomeric purity of rocuronium bromide API has been developed and validated. The compounds were well separated isocratically on an Inertsil silica column using a mobile phase consisting of Solution A (Sodium Perchlorate): Solution B (ammonium chloride +Ammonia) 75:25 with PDA detector. Retention time for rocuronium bromide was found to be 10.7 respectively. The study showed that the reverse phased liquid chromatography is sensitive and selective for detecting stereo isomeric purity of rocuronium bromide using the single mobile phase.**

***Keywords*— stereo isomeric purity, Rocuronium bromide, neuro muscular blocking agent, RP-HPLC, PDA detector**

I. INTRODUCTION

Rocuronium Bromide 1-[17β-(acetyloxy)-3α-hydroxy-2β-(4-morpholinyl)-5α-androstan-16β-yl]-1-(2-propenyl) pyrrolidinium bromide shown in Fig.1 is a neuro muscular blocking agent. It is completely soluble in water and stable under normal conditions.

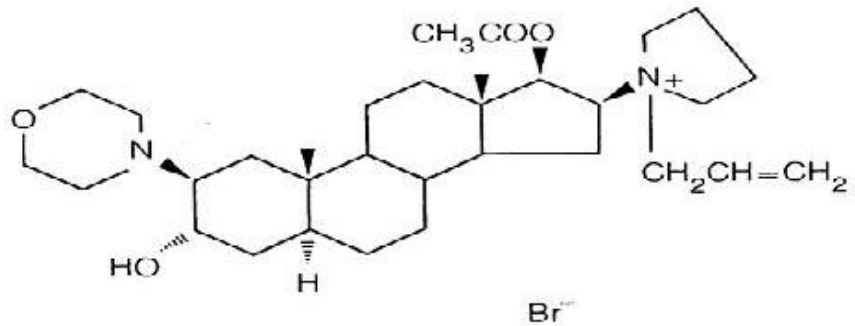

Fig. 1 Structure of Rocuronium Bromide

In general, neuromuscular blockers prevent the transmission of electrical impulses to the muscle by altering the normal interaction of acetylcholine with the postsynaptic cholinergic receptor. Rocuronium, an analog of vecuronium, is an amino steroid neuromuscular blocker which exerts its action through competitive inhibition of the cholinergic receptor at the motor end-plate of the myoneural junction. Rocuronium blocks the effect of both the small quantities of acetylcholine that maintain muscle tone and the large quantities of acetylcholine that produce voluntary muscle contraction, but does not alter the resting electrical potential of the motor endplate or cause muscle contraction.

II. EXPERIMENTAL WORK

A. Method Development:

The chromatographic procedure may be carried out using a High Performance Liquid Chromatogram equipped with an UV detector.

Solution-A: Dissolve 10.0 g of sodium per chlorate monohydrate in 6.0 ml of water and make up to 1000 ml with Acetonitrile.
Solution-B: Weigh about 2.0 g of ammonium chloride, add 8 ml of ammonia and make up to 1000 ml with methanol.
Mobile phase: Solution-A: Solution-B→75:25

Column: Inertsil Silica (250 mm x 4.6 mm, 5µm) or equivalent
Flow rate: 0.5 ml/min
Injection volume: 20 µl
λ_{max}: 215 nm
Sample concentration: 2.0 mg/ml
Diluent: Accurately pipette out 1.0 ml of 1N hydrochloric acid into a 1000 ml volumetric flask and make up to the volume with Acetonitrile.
System Suitability Solution:
Dissolve accurately weighed quantities of Rocuronium bromide impurity - C (Quarternary salt of Diol) and Rocuronium bromide impurity - A (mono acetate) and Rocuronium bromide working standard with diluent to obtain a solution having known concentration of about 0.5 mg/ml, 0.5 mg/ml and 1.0 mg/ml each respectively.
Excipient stock:
Accurately weigh about 83.13 mg of sodium acetate trihydrate and 42.5 mg of sodium chloride in a 25 ml volumetric flask, dissolve and dilute to volume with water.
Excipient solution:
Accurately pipette out 2.0 ml of the excipient stock solution into a 10.0 ml volumetric flask and make up to the volume with the diluent.
Standard solution:
Weigh 50 mg of Rocuronium Bromide standard into a 25 ml volumetric flask, dissolve and dilute to volume with the diluent.
Sample solution:
Accurately pipette out 2.0 ml of the injection into a 10.0 ml volumetric flask and make up to the volume with the diluent.
optimized method:
Diluent: Accurately pipette out 1.0 ml of 1N hydrochloric acid into a 1000 ml volumetric flask and make up to the volume with Acetonitrile.
Column: Inertsil silica column (250 x 4.6mm) 5µ
Mobile phase: Solution A: Solution B (75:25)
Injection Volume: 20 µl
Flow rate: 0.5ml/min
Column temp: 25^{0}C
Detection wavelength: 215nm

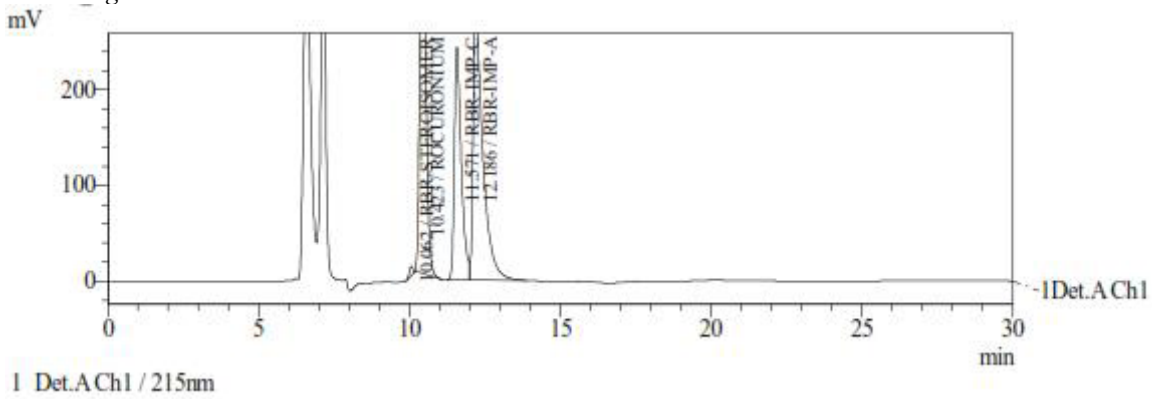

Fig. 5 Chromatogram of optimized method

Inference: All three peaks were separating with reasonable retention times and the resolution between diol and RBR was good.
B. Standards, Materials and Equipment:

High performance liquid chromatography equipped with Isocratic pump, UV detector, auto sampler and column heating oven.

TABLE 1 LIST OF STANDARDS

Rocuronium bromide	Working/ Reference standard
Rocuronium bromide stereo isomer	Working/ Reference standard
Rocuronium bromide impurity-A	Working/ Reference standard
Rocuronium bromide impurity-C	Working/ Reference standard

TABLE 2 MATERIALS

Material	**Grade/Make**
Sodium per chlorate monohydrate	GR, Merck or equivalent
Ammonium chloride	GR, Merck or equivalent
Ammonia	GR, Merck or equivalent
Acetonitrile	HPLC, Merck or equivalent
Methanol	HPLC, Merck or equivalent

TABLE 3 HPLC CONDITIONS

Parameter	Condition
Column	Inertsil Silica (250 mm x 4.6 mm, 5µm) or equivalent
Flow rate	0.5 mL/min
Wave length	215 nm
Injection volume	20 µL
Sample concentration	30 minutes
Sample concentration	2.0 mg/Ml

System Suitability and Sample Preparation:

Diluent: Acetonitrile: water ===> 90:10

System Suitability Solution:

Prepare a solution containing Rocuronium bromide impurity - C (Quaternary salt of Diol), Rocuronium bromide impurity – A (mono acetate), stereoisomer of Rocuronium bromide and Rocuronium bromide working standard with diluent to obtain a solution having known concentration of about 0.5 mg/ml, 0.5 mg/ml, 0.02 mg/ml and 1.0 mg/ml each respectively.

Sample Solution:

Weigh 50 mg of Rocuronium Bromide sample into a 25 mL volumetric flask, dissolve and dilute to volume with the diluent.

Procedure:

Separately inject 20 µL each of the diluent, system suitability solution, and the sample solution (duplicate injections) into the chromatograph, record the chromatograms, and measure only the peak responses of Rocuronium and the stereo isomeric impurity in sample solution. Report the stereo isomeric purity of the Rocuronium bromide in sample solution by area normalization mode.

System Suitability Criteria:

1. The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0.
2. The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0.
3. The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0.

Note: The retention time of the stereo isomeric impurity is about 10.7 minutes and retention time of Rocuronium bromide is about 11.2 minutes.

Note: To identify the stereo isomeric peak in the test solution, compare and use the RRT's of other stereoisomer obtained from the system suitability solution chromatogram

Calculation: The stereo isomeric purity of Rocuronium bromide is

$$\frac{\text{Average area of Rocuronium bromide}}{\text{Total average area}} \times 100$$

Total average area = sum of the average areas of Rocuronium bromide and stereo isomeric Impurity.

III. METHOD VALIDATION

A) *System Suitability & Control Sample Analysis*

Prepared and injected system suitability solution and evaluated system suitability parameters as per test method. Stereo isomer content in Rocuronium bromide API sample was determined. Acceptance criteria and results for system suitability & control sample analysis were presented in Table 4

TABLE 4 ACCEPTANCE CRITERIA AND RESULTS FOR SYSTEM SUITABILITY AND CONTROL SAMPLE

Acceptance criteria	Result
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0	3.054
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0	1.394
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	1.102
Report the control sample stereo isomeric purity results	Not detected hence not reported

B) *Method Precision (Repeatability)*

i) *Method Precision on Control Sample:*

Repeatability was determined by analyzing six different control sample preparations from same drug substance lot at 100 % level of method concentration. Prepared the Rocuronium bromide control sample solution six times, injected each solution once and analyzed as per method. The system suitability results were presented in Table 5 and precision results were presented in Table 6. Acceptance criteria and results for control sample precision study were presented in Table 7

TABLE 5 SYSTEM SUITABILITY AND ACCEPTANCE CRITERIA AND RESULTS FOR METHOD PRECISION ON CONTROL SAMPLE

Acceptance criteria	Result
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0	Resolution : 3.117
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0	Resolution: 1.320
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	Resolution: 1.332

TABLE 6 CONTROL SAMPLE PRECISION RESULTS

# of preparations	Stereoisomer content (%)

1	ND
2	ND
3	ND
4	ND
5	ND
6	ND
Average	NA
Std dev	NA
%RSD	NA

TABLE 7 ACCEPTANCE CRITERIA AND RESULTS FOR METHOD PRECISION ON CONTROL SAMPLE

Acceptance criteria	**Result**
System should meet the system suitability criteria.	Complies
% RSD for the content of Rocuronium bromide stereo isomer from 6 control sample preparations should be not more than 15.0.	Stereoisomer not detected hence not reported

ii) Method Precision on Spiked Sample:

Repeatability will be determined by analyzing six different control sample spiked preparations. The sample will be prepared by spiking Rocuronium bromide stereo isomer at specification limit (NMT 0.1%) with respect to test concentration of 2 mg/mL. A single injection of each sample preparation will be performed. %content of Rocuronium bromide stereo isomer will be determined for each of the sample preparation. The % relative standard deviation for Rocuronium bromide stereo isomer % content for 6 preparations will be determined. Spiked precision results were taken from range study. The system suitability results were presented in Table 8 and spiked sample precision results were presented in Table 9. The acceptance criteria & results of spiked precision are presented in Table 10.

TABLE 8: ACCEPTANCE CRITERIAAND RESULTS FORSYSTEM SUITABILITY– SPIKED SAMPLE ANALYSIS

Acceptance criteria	**Result**
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0.	Resolution : 3.199
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0.	Resolution: 1.082
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0.	Resolution: 1.283

TABLE 9: SPIKED PRECISION RESULTS

# of preparations	**% Content**
1	0.090
2	0.087
3	0.089
4	0.090
5	0.088
6	0.093
Average	0.089
Std dev	0.0022
%RSD	2.4

TABLE 10: ACCEPTANCE CRITERIA AND RESULTS FOR SPIKED PRECISION

Acceptance criteria	**Result**
System should meet the system suitability criteria.	Complies
% RSD for %content of Rocuronium bromide stereo isomer results from six different spiked sample preparations should be NMT 15.0.	%RSD : 2.4

C) *Intermediate Precision (Ruggedness)*

In order to demonstrate ruggedness, six different spiked sample preparations were prepared as in the 'Method precision on spiked sample' study and analyzed by another chemist. The two individual precision studies were done by the two chemists and they used different system suitability solution preparations, different sample preparations, different mobile phase lots, different column lots and different HPLC systems on different days. % content of Rocuronium bromide stereo isomer for each preparation of spiked samples was calculated. % RSD for % content of Rocuronium bromide stereo isomer 6 preparations and % RSD for % contents of Rocuronium bromide stereo isomer of 12 determinations of spiked samples (6 from spiked Precision study and 6 from intermediate Precision study) were calculated. The system suitability results were presented in Table 11 and Intermediate precision results were presented in Table 12. Ruggedness results were presented in Table 13. The acceptance criteria & results are presented in Table 14.

TABLE 11: SYSTEM SUITABILITY RESULTS FOR INTERMEDIATE PRECISION STUDY

Acceptance criteria	Result
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0.	Resolution : 2.863
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0.	Resolution: 1.571
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0.	Resolution: 1.155

TABLE 12: INTERMEDIATE PRECISION RESULTS

# of preparations	% Content
1	0.099
2	0.096
3	0.095
4	0.096
5	0.098
6	0.097
Average	0.0966
Std dev	0.001
%RSD	1.450

TABLE 13: RUGGEDNESS RESULTS (12 DETERMINATIONS)

Parameter	Content (%)
Precision study-1results	0.090
	0.087
	0.089
	0.090
	0.088
	0.093
Precision study-2 results	0.099
	0.096
	0.095
	0.096
	0.098
	0.097
Average	0.1
SD	0.0042
% RSD	4.5

TABLE 14: ACCEPTANCE CRITERIA AND REULTS

Acceptance criteria	Results
System should meet the system suitability criteria.	Complies
% RSD for % content of Rocuronium bromide stereo isomer results from six different spiked sample preparations should be NMT 15.0	%RSD : 1.5
% RSD of % content of Rocuronium bromide stereo isomer for 12 determinations (method precision on spiked sample & Intermediate precision) should be NMT 15.0	% RSD : 4.4

D) *Linearity of Detector Response:*

Linearity was performed to assess whether a single point calibration would provide sufficient accuracy over the intended operating range of the method. Standard linearity solutions were prepared from a stock solution of Rocuronium bromide stereo isomer and utilizing seven single preparations over the specified range to evaluate the linearity. Seven concentration levels were analyzed over a range of LOQ to 200 % of the specification level (NMT 0.1%) with respect to method concentration 2 mg/mL. One preparation at each level was injected once. Individual data points are reported. A plot of concentration versus area response is presented in the validation report. The concentrations were reported to four significant figures and areas were reported as whole number. Linear regression analysis (without forcing through the origin) was performed on the data. The correlation coefficient (r^2), slope of the regression line and % Y-intercept are calculated and reported. The solution preparations of linearity study are described in Table 15.

TABLE 15: LINEARITY SOLUTIONS PREPARATION

Solution	Preparation
Rocuronium bromide stereo isomer standard stock solution (1.0mg/mL)	Accurately weighed 2.324 mg of Rocuronium bromide stereo isomer standard into a 2 mL volumetric flask, dissolved in diluent and diluted to the mark with diluent.
Rocuronium bromide stereo isomer standard solution-1 (0.01mg/mL)	Accurately pipette out 1 mL of Rocuronium bromide stereo isomer standard stock solution into a 100 mL volumetric flask, diluted to volume with diluent.

Linearity level	Conc. (µg/mL) with assay correction	Preparation
LOQ	0.2589	Accurately pipette out 253.5µL of Rocuronium bromide stereo isomer standard solution-1 into a 10mL volumetric flask, diluted to volume with diluent.
50%	1.0214	Accurately pipette out 1.0 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.
75%	1.5321	Accurately pipette out 1.5 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.
100%	2.0428	Accurately pipette out 2.0 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.
125%	2.5535	Accurately pipette out 2.5 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.
150 %	3.0642	Accurately pipette out 3.0 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.
200 %	4.0856	Accurately pipette out 4.0 mL of Rocuronium bromide stereo isomer standard solution-1 into a 10 mL volumetric flask, diluted to volume with diluent.

The system suitability results are presented in Table 16 and the results of linearity study are presented in Table 17 and acceptance criteria & Results are presented in Table 18.

TABLE 16: SYSTEM SUITABILITY RESULTS FOR LINEARITY STUDY

Acceptance criteria	Results
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0	Resolution : 3.106
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0	Resolution: 1.625
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	Resolution: 1.067

TABLE 17: RESULTS FOR LINEARITY OF DETECTOR RESPONSE STUDY

% Concentration with respect to method concentration	Concentration (µg/mL)	Area of Rocuronium stereoisomer
LOQ	0.2589	2794
50	1.0214	8687
75	1.5321	12795
100	2.0428	17431
125	2.5535	22630
150	3.0642	27595
200	4.0856	36029
Intercept		-152.4989
% Y-intercept		-0.9
Correlation coefficient		0.99903

TABLE 18: ACCEPTANCE CRITERIA AND RESULTS

Acceptance criteria	Results
System should meet the system suitability criteria.	Complies
The correlation coefficient (r^2) should be not less than 0.990 for Rocuronium bromide stereo isomer (report the value of r as follows: all of the repeating 9s plus the next digit, truncated, not rounded).	Correlation coefficient (r^2) :0.9990
%Y-Intercept value should be within ±5.0 to nominal concentration (100% level).	% Y-intercept: -0.9

E) *Detection Limit and Quantification*

a) *Detection Limit:*

The detection limit is determined by the analysis of a standard solution with concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected but not necessarily quantifiable. Determine the limit of detection of Rocuronium bromide stereo isomer based on signal to noise ratio. Derive the approximate concentration which will give Signal to Noise ratio of about 3 for limit of detection.

b) *Quantification Limit:*

The limit of detection and Limit of quantitation can also be determined by using Slope method. The LOD and LOQ values of Rocuronium bromide stereo isomer can be determined by injecting the linear standard solutions and determine the values using the following equation as given below.

$$LOD = \frac{3.3 \times S_a}{b}$$

$$LOQ = \frac{10 \times S_a}{b}$$

Where:

Sa= Standard error of the predicted Y value for each x in the regression

b = Slope of the calibration curve from the regression equation

For LOD/LOQ determination by the slope method, a series of linearity standard solutions of Rocuronium bromide stereo isomer from the expected minimum level of detection to 0.1 %with respect to the test concentration (2 mg/mL) were prepared and

injected. The below Table 19 describes the solution preparations of Rocuronium bromide stereo isomer for deriving the LOD/LOQ values by the slope method.

TABLE 19: LINEARITY SOLUTIONS FOR DETERMINATION OF LOD/LOQ VALUES

Solution	Preparation
Rocuronium bromide stereoisomer standard stock solution (0.1124 mg/mL)	Accurately weighed 5.618 mg of Rocuronium bromide stereo isomer standard into a 50 mL volumetric flask, dissolved and diluted to volume with diluent.

Level (%)	Conc. (µg/mL) with assay correction	Preparation
0.1	1.9753	Accurately pipette out 0.2 mL of Rocuronium bromide stereoisomer standard stock solution into a 10 mL volumetric flask, diluted to volume with water.
0.075	1.4815	Accurately pipette out 0.15 mL of Rocuronium bromide stereoisomer standard stock solution into a 10 mL volumetric flask, diluted to volume with water.
0.05	0.9876	Accurately pipette out 0.1 mL of Rocuronium bromide stereoisomer standard stock solution into a 10 mL volumetric flask, diluted to volume with water.
0.025	0.4938	Accurately pipette out 0.05 mL of Rocuronium bromide stereoisomer standard stock solution into a 10 mL volumetric flask, diluted to volume with water.
0.01	0.1975	Accurately pipette out 1.0 mL of 0.1% Rocuronium bromide stereoisomer standard solution into a 10 mL volumetric flask, diluted to volume with water.
0.005	0.0988	Accurately pipette out 1.0 mL of 0.05% Rocuronium bromide stereoisomer standard solution into a 10 mL volumetric flask, diluted to volume with water.
0.001	0.0198	Accurately pipette out 1.0 mL of 0.01% Rocuronium bromide stereoisomer standard solution into a 10 mL volumetric flask, diluted to volume with water.

The system suitability results are presented in Table 20and the LOD & LOQ results are presented in Table 21. The LOD & LOQ study acceptance criteria & results are presented in Table 22.

TABLE 20 SYSTEM SUITABILTY RESULTS FOR LOD & LOQ STUDY

Acceptance criteria	Results
The resolution factor between the peaks due to Rocuronium Bromide and Impurity C is not less than1.0	Resolution : 3.060
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0	Resolution: 1.626
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	Resolution: 1.090

TABLE 21: RESULTS FOR LOD/ LOQ

% Concentration with respect to method concentration	Concentration (µg/mL)	Area of Rocuronium stereoisomer
0.001	0.0198	675
0.005	0.0988	1242
0.01	0.1975	2407
0.025	0.4938	4706
0.05	0.9876	10155
0.075	1.4815	15188
0.1	1.9753	19957
Steyx		257.6924
Slope		9969.2932
LOD (in µg/mL)		0.0853
LOQ (in µg/mL)		0.2585
LOD (%)		0.004
LOQ (%)		0.013

TABLE 22 ACCEPTANCE CRITERIA AND RESULTS

Acceptance criteria	Results
System should meet the system suitability criteria.	Complies
Report LOD and LOQ values in µg/mL or mg/mL or in % w.r.t test concentration.	LOD : 0.004% LOQ : 0.013 %

F) *Precision at LOQ:*

Prepared a solution of Rocuronium bromide stereo isomer at a concentration in mg/mL or µg/mL equal to the estimated QL and the LOQ solution was analyzed according to the method and replicating the analysis for 6 times. % RSD value of the Rocuronium bromide stereo isomer areas was calculated. The system suitability results were presented in Table 23 and the precision at LOQ results are presented in Table 24. The precision at LOQ study acceptance criteria & results are presented in Table 25.

TABLE 23 SYSTEM SUITABILITY RESULTS FOR PRECISION AT LOQ STUDY

Acceptance criteria	Results
The resolution factor between the peaks due to Rocuronium Bromide and Impurity C is not less than1.0	Resolution : 3.106
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0	Resolution: 1.625
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	Resolution: 1.067

TABLE 24 RESULTS FOR PRECISION AT LOQ

#of injections	Area of Rocuronium stereoisomer
1	2744
2	2715
3	2738
4	2791
5	2770
6	2804
Average	2760
Std dev	33.9451
%RSD	1.2

TABLE 25 ACCEPTANCE CRITERIA AND RESULTS

Acceptance criteria	Results
System should meet the system suitability criteria.	Complies
The % RSD value of the Rocuronium bromide stereo isomer peak areas from 6 replicates of LOQ solution should be not more than 15.0.	% RSD : 1.2

G) *Selectivity (Specificity& Stability Indicating Characteristics):*

i) *Specificity:*

Specificity was evaluated to ensure that no other compounds that may be present interfere appreciably with the quantitation of Rocuronium bromide stereo isomer. To demonstrate that the peaks due to blank, Rocuronium and Rocuronium bromide impurities/degradation compounds do not interfere with the quantitation of Rocuronium bromide stereo isomer, drug substance, Rocuronium bromide stereo isomer and Rocuronium bromide identification CRS was analyzed individually and the drug substance was spiked with Rocuronium bromide stereo isomer at the specification level of Rocuronium bromide stereo isomer and compared their retention times to establish separation. The system suitability results were presented in Table 26, specificity results were presented in Table 27 Acceptance criteria and results for specificity study were presented in Table 28.

TABLE 26 SYSTEM SUITABILITY RESULTS FOR SPECIFICITY STUDY

Acceptance criteria	Results
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0.	Resolution : 2.844
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than1.0.	Resolution: 1.170
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0.	Resolution: 1.046

TABLE 27 RESULTS OF SPECIFICITY STUDY

S.No	Sample	Retention time (mins)	Remarks
1	Blank	6.306, 6.507, 6.899 & 7.370	No interference with Rocuronium stereoisomer peak
2	System suitability solution	Stereo isomer: 10.392	No interference of blank and other related compounds with analyte peak
		Rocuronium : 10.759	
		Impurity-C : 11.878	
		Impurity-A: 12.462	
3	Rocuronium peak identification CRS	Impurity-B: 9.525	No interference of blank and other related compounds with analyte peak
		Impurity-F: 9.654	
		Impurity-H: 10.085	
		Rocuronium : 10.638	
		Impurity-C : 11.904	
		Impurity-A: 12.844	
		Impurity-G : 19.805	

4	Rocuronium bromide control sample	Rocuronium : 10.714	No interference of blank and other related compounds with analyte peak
5	Rocuronium bromide sample spiked with stereoisomer	Rocuronium stereoisomer: 10.379 Rocuronium : 10.674	No interference of blank and other related compounds with analyte peak

TABLE 28 ACCEPTANCE CRETERIA AND RESULTS

Acceptance criteria	**Results**
System should meet system suitability criteria	Complies
Peaks in the chromatograms due to blank and individual impurities should not interfere with the quantitation of Rocuronium bromide stereo isomer.	Complies

ii) *Stability-Indicating Characteristics:*

Stability indicating characteristics of the method was demonstrated by its ability to separate Rocuronium bromide stereo isomer from Rocuronium bromide and its related impurities. The control, heat, acid, base and peroxide stressed samples prepared (refer Table 29) were used for this study. These forced degradation samples were analyzed according to the analytical method RBRAPI-AM-02-00. Spectral match was not performed because stereo isomer not detected in control and stressed samples.

TABLE 29 FORCED-DEGRADATION SAMPLE PREPARATIONS

Sample	**Preparation**
Control	Weighed accurately 20.34 mg of Rocuronium bromide API sample in to a 10 mL volumetric flask, dissolved in diluent and diluted to the volume with the diluent.
Dry heat	Taken about 50 mg of Rocuronium bromide API sample in a glass stopper flask/bottle and subjected to heat stress by placing the flask/ bottle in an oven set at 80oC for 2 days. After 2 days it was taken out and allowed to cool to room temperature. Weighed accurately 20.17 mg of Rocuronium bromide dry heat sample in to a 10 mL volumetric flask, dissolved in diluent and diluted to the volume with the diluent.
Acid stress	Weighed accurately about 40.58 mg of Rocuronium bromide API sample into a 20 mL volumetric flask, dissolve in10 mL of 0.1 N Hydrochloric acid and kept the sample at room temperature for 2 hours. After 2 hours, adjusted the pH of solution to 6.942 with 1N NaOH and quantitatively diluted to the volume with diluent.
Base stress	Weighed accurately about 40.86 mg of Rocuronium bromide API sample into a 20 mL volumetric flask, dissolve in 10 mL of 0.1 N sodium hydroxide and kept the sample at room temperature for 2 hours. After 2 hours, adjusted the pH of solution to 6.912 with 1N HCl and quantitatively diluted to the volume with diluent.
Peroxide Stress	Weighed accurately about 40.55 mg of Rocuronium bromide API sample into a 20 mL volumetric flask, dissolved in 10 mL of 3.0 % hydrogen peroxide and kept the sample at room temperature for 2 hours. After 2 hours quantitatively make up the volume to 20 mL with diluent.

The system suitability results are presented in Table 30, the stress study results are presented in Table 31 respectively.

TABLE 30 SYSTEM SUITABILITY RESULTS FOR STRESS STUDY

Acceptance criteria	**Results**
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0	Resolution : 2.844
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than 1.0	Resolution: 1.170
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0	Resolution: 1.046

TABLE 31 STRESS STUDY RESULTS OF ROCURONIUM BROMIDE

S.No	**Condition**	**Rocuronium bromide stereo isomer (%)**
1	Control	ND
2	Acid	ND
3	Heat	ND
4	Base	ND
5	Peroxide	ND

TABLE 32 ACCEPTANCE CRITERIA & RESULTS – STRESS STUDY

Acceptance criteria	**Result**
The system should meet system suitability criteria.	Complies
An increase in the stereo isomer content should be observed for the samples where degradation is expected.	Stereo isomer not detected hence not reported

H) *Range (Accuracy, Precision & Linearity of the Test Method)*

Recovery samples were prepared spiking Rocuronium bromide stereo isomer standard solution to Rocuronium bromide API spanning from LOQ to 200% of specification level (NMT 0.1 % for Rocuronium bromide stereo isomer) with respect to the test concentration of Rocuronium bromide 2 mg/mL(100% test concentration). Three preparations were made at each level except at the LOQ, 100% and 200% levels, where six preparations were made. Each solution was injected once and analyzed according to the test method (RBR-API-AM- 02-00). The percentage recovery of Rocuronium bromide stereo isomer was calculated for the individual preparations at each level and a mean of the recovery was determined based on the un-rounded data and reported to one decimal place. The percent RSD was calculated for each level from the un-rounded results and reported to one decimal place. The system suitability results were presented in Table 33 and accuracy (recovery) results were presented in Table 34.

% Recoveries will be calculated using Equation 1 to5.

Equation 1:

Amount Present

Content of Rocuronium bromide stereo isomer in control sample by area normalization mode [A] in %:

$$\% \text{ Amount present [A]} = \frac{AC}{\text{Total average area of control sample}} \times 100$$

AC =Average area of Rocuronium bromide stereo isomer in control sample

Equation 2:

Amount Found

Amount of Rocuronium bromide stereo isomer in the spiked sample by area normalization mode [B] in %:

$$\% \text{ Amount found [B]} = \frac{AS}{At} \times 100$$

As = Area of Rocuronium bromide stereoisomer in spiked sample

At = Sum of area of Stereo isomer peak and Rocuronium peak in spiked sample

Equation 3:

Amount Recovered

Amount of Rocuronium bromide stereo isomer recovered = [B] – [A]

Equation 4:

Amount Added

$$\% \text{ Amount added} = Cs \times \frac{Vs}{10} \times \frac{P}{\text{Method concentration (mg/mL)}}$$

Cs= Concentration of Rocuronium bromide stereoisomer in standard stock solution-2 (mg/mL)

VS= Volume of Rocuronium bromide stereoisomer standard stock solution-2 taken to make range solution (mL).

P = Purity /assay of stereo isomer

Equation 5:

Percent Recovery Calculation

$$\% \text{ of Recovery} = \frac{\text{Amount recovered}}{\text{Amount added}} \times 100$$

TABLE 33: SYSTEM SUITABILITY RESULTS FOR RANGE STUDY

Acceptance criteria	**Results**
The resolution factor between the peaks due to Rocuronium Bromide and Impurity-C is not less than1.0	Resolution : 3.199
The resolution factor between the peaks due to Impurity-C and Impurity-A is not less than 1.0	Resolution: 1.082
The resolution factor between the peaks due to stereoisomer and Rocuronium is not less than 1.0.	Resolution: 1.283

TABLE 34 RECEVORY RESULTS

Level	Amount added (%)	Amount recovered (%)	% Recovery	Average	%RSD
LOQ	0.0101	0.0094	93.4	96.4	3.5
	0.0101	0.0096	95.2		
	0.0101	0.0101	100.0		
	0.0101	0.0095	93.7		
	0.0101	0.0096	94.9		
	0.0101	0.0102	101.2		
50 %	0.0505	0.0445	88.1	88.8	1.9

	0.0505	0.0443	87.6		
	0.0505	0.0458	90.7		
100%	0.1010	0.0897	88.8	88.4	2.4
	0.1010	0.0867	85.8		
	0.1010	0.0892	88.3		
	0.1010	0.0901	89.2		
	0.1010	0.0876	86.7		
	0.1010	0.0929	92.0		
150%	0.1516	0.1551	102.3	103.3	0.9
	0.1516	0.1565	103.2		
	0.1516	0.1580	104.2		
200%	0.2021	0.2108	104.3	104.0	1.5
	0.2021	0.2101	104.0		
	0.2021	0.2058	101.9		
	0.2021	0.2101	104.0		
	0.2021	0.2155	106.6		
	0.2021	0.2085	103.2		

Linearity of the Test Method

The Linearity graph had been plotted between the 'average amounts of Rocuronium bromide stereo isomer added in %' versus 'average amount of Rocuronium bromide stereo isomer recovered in %' in accuracy from LOQ to 200% of specification limit (NMT 0.1%) with respect to the test concentration of 2.0 mg/mL of Rocuronium bromide. The correlation coefficient was calculated and reported. The results for linearity of the Rocuronium bromide stereo isomer are reported in Table 35. The plot of average amount added (%) versus average amount recovered (%) was presented in Figure 1.Acceptance criteria and results were presented in Table 36.

TABLE 35 LINEARITY OF ROCURONIUM BROMIDE

Level	Average amount added (%)	Average amount recovered (%)
LOQ	0.0101	0.0097
50%	0.0505	0.0449
100%	0.1010	0.0894
150%	0.1516	0.1565
200%	0.2021	0.2102
Correlation coefficient (r^2)	r^2= 0.9968	

TABLE 36 ACCEPTANCE CRITERIA & RESULTS-RANGE STUDY

Acceptance criteria	**Results**
System should meet the system suitability criteria.	Complies
% Recovery at each accuracy level (individual preparations and mean) must be within 80.0 – 120.0% of the theoretical concentration.	Individual recovery: 85.8% - 106.6% Mean recovery: 88.4% - 104.0%
% RSD of % recoveries at each accuracy level should be not more than 15.0.	0.9 - 3.5
The correlation coefficient (r^2) for Rocuronium bromide stereo isomer should be NLT 0.990 (report the value of r^2 as follows: all of the repeating 9s plus the next digit, truncated, not rounded).	r^2= 0.996

SUMMARY:

The analytical method validation report RBR-API-AVR-10-00demonstrated system precision, system suitability, control sample analysis, precision on control sample (repeatability), spiked precision, intermediate precision (ruggedness), linearity of detector response, selectivity (Specificity& stability indicating characteristics), detection limit, quantification limit, precision at LOQ and range (accuracy, precision & linearity of test method) of the test method. The method was found to be accurate, precise, and linear over range of LOQ to 200% of the specification level (NMT 0.1%) with respect to the method concentration 2 mg/mL. The method was found to be both repeatable and rugged. The method was also found to be specific and selective to Rocuronium bromide stereo isomer. The method was found to be linear for Rocuronium bromide stereo isomer over the range of LOQ to 200% of the specification level (NMT 0.1%) with respect to the method concentration-2 mg/mL. The limit of detection and limit of quantification was established for Rocuronium bromide stereo isomer. Precision was established for Rocuronium bromide stereo isomer at the quantification level.

IV. CONCLUSION

The results of this validation study indicate that the analytical method for the determination of stereo isomeric purity of Rocuronium bromide API by HPLC method is found to be accurate, precise, and linear over range of LOQ to 200% of the

specification level (NMT 0.1%) with respect to the method concentration 2 mg/mL. The method was found to be both repeatable and rugged. The method was also found to be specific and selective to Rocuronium bromide stereo isomer. The method was found to be linear for Rocuronium bromide stereo isomer over the range of LOQ to 200% of the specification level (NMT 0.1%) with respect to the method concentration 2 mg/mL. The limit of detection and limit of quantification was established for Rocuronium bromide stereo isomer. Precision was established for Rocuronium bromide stereo isomer at the quantification level.

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