CHAPTER - 3

ANALYTICAL METHODS

3.1 INTRODUCTION

UV spectrophotometric method is a robust analytical technique for the routine analysis of bulk samples, formulations and dissolution. The objective of the present study was to develop simple, accurate, precise analytical method with the optimal detection range for drug, formulatedproduct and in-vitro release rate studies. The medium used was pH 7.4 phosphate buffer saline (λ_{max}-272 nm) (1). The methods developed was validated according to ICH guidelines and USP requirements. Adequate statistical tests on validation data were performed.

Till date, only two methods of LC-MS have been described to detect TAP in biological matrices urine and oral fluid (2) and two HPLC methods for the determination of TAP in Tablet dosage forms has been reported (3). However, there have been no studies on an HPLC-MS / MS method for the detection of plasma TAP (4). Therefore, there is a need to develop an LC-MS / MS method for detecting Tapentadol in rabbit plasma.

3.2 UV SPECTROPHOTOMETRIC METHOD DEVELOPMENT

3.2.1 Instruments

A Beckman DU 640 UV-visible double beam spectrophotometer connected to a computer loaded with software is used. For Instrument it has an automatic wavelength accuracy of 0.1 nm and adapted quartz cells of 10 mm path length.

3.2.2 Materials

TapentadolHCl was obtained from the Medicinal chemistry department of Torrent Pharma (Source: Symed labs, India), other chemicals and reagents used were of analytical quality.

3.2.3 Analytical method development

Different media were investigated to develop a UV-spectrophotometric method suitable for candidate drug analysis. For the selection of media, the criteria used were the sensitivity of the method, the ease of sample preparation, the solubility of the drug, and the cost of solvents and the applicability of the method for various purposes.

3.2.4 Calibration standards

A stock solution of 10000 µg /ml in methanol was prepared by dissolving 100 mg of drug in 10 ml of methanol. Secondary stock solutions were prepared by taking aliquots of stock solutions were transferred into a series of 10 ml standard flasks and volumes were made up with methanol. For the preparation of different concentrations, 0.1 ml of working stock solution was transferred into a series of standard 10 ml flasks and volumes were made with respective media.

Six different concentrations were prepared in the range of 20-100 µg/ ml of the drug in the phosphate buffered saline solution for the standard plot. The drug was estimated at 272 nm. The calibration data are presented in the table

Table 3.1: Calibration data of the developed method of Tapentadol

Conc. (µg /ml)	Average*(±S.D.[a])	%RSD[b]
20	0.202±0.0011	0.545124
40	0.322±0.0003	0.089447
50	0.406±0.0018	0.448113
70	0.587±0.0043	0.725062
80	0.670±0.0022	0.325097
100	0.852±0.0008	0.093864

*Average of triplicate determination
a=Standard deviation,
b=Relative standard deviation

3.2.5 Analytical method validation

3.2.5.1 Accuracy

As part of the determination of the accuracy of the proposed methods, different levels of drug concentrations (LQC, MQC, and HQC in both media) were prepared from an independent stock solution and analyzed (N = 9). Precision was evaluated as the relative error percentage and the mean percent recovery.

3.2.5.2 Precision

Repeatability was determined using different levels of drug concentrations (the same concentration levels taken in the precision study), prepared from an independent stock solution and the (days) and intra-day variations (N = 9) were analyzed.

3.2.5.3 Linearity

To establish the linearity of the proposed method, six separate solutions of the drug 20-100 µg ml-1 were prepared in the phosphate buffer saline from the stock solutions and analyzed. A least squares regression analysis was performed for the data obtained.

3.2.5.4 Detection limit (DL) and quantitation limit (QL)

The DL and QL by the proposed methods were determined using calibration standards. DL and QL were calculated as 3.3r / S and 10r / S, respectively, where S is the slope of the calibration curve and r is the standard deviation of the intersection and the regression equation.

3.2.5.5 Specificity

Drug solutions (30 µg ml^{-1}) were prepared in the media selected together with and without common excipients (polysorbate 80, polyvinylpyrrolidone). All solutions were scanned from 450 to 200 nm at a speed of 1200 nm min^{-1} and were found to change in absorbance at respective wavelengths.

3.3 RESULTS AND DISCUSSION

3.3.1 Analytical method development

The λmax of the drug in phosphate buffer saline was found to be 272 nm. The apparent molar absorptivity of the drug was found to be 2.62 x 104 1 mol^{-1} cm^{-1}.

3.3.2 Calibration curve:

In the phosphate buffer, the linear regression equation obtained was: absorbance at 272nm = [0.0083 × concentration in µg ml–1] + 0.01; with a regression coefficient of 0.9945.

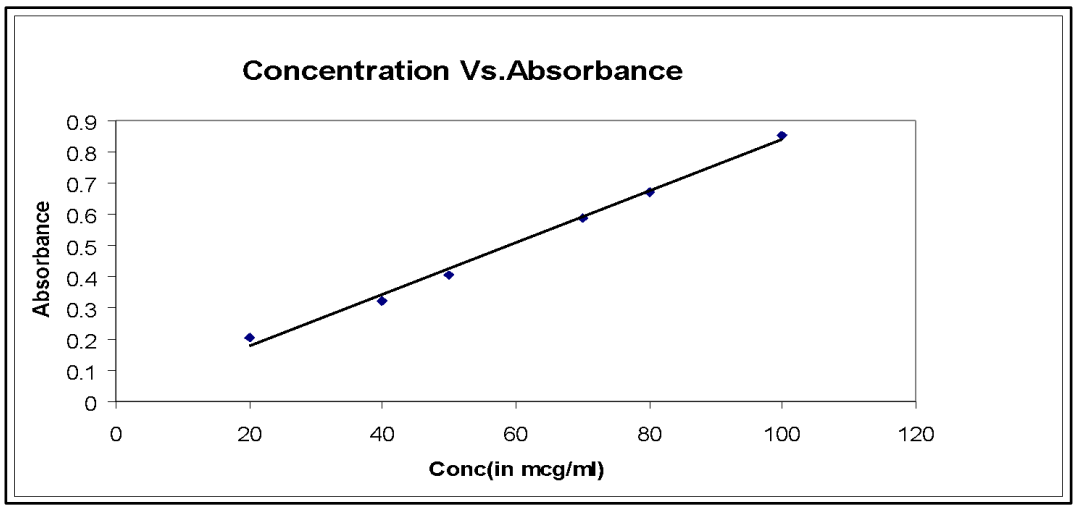


Figure 3.1: Calibration curve of Tapentadol in phosphate buffer

3.4 ANALYTICAL VALIDATION:

3.4.1 Accuracy

Precision ranged from -0.85 to 2.43%. The excellent average recovery values (almost 100%) and their low standard deviation values (S.D. <1.2) represent accuracy. In phosphate buffer,the mean recovery percentage (RSD) for lower, intermediate and higher concentrations was found to be 97.58 (1.64), 99.16 (1.706) and 98.72 (1.56) respectively. These results revealed that any small change in drug concentration in the solution can be accurately determined by these proposed methods.

Table 3.2: Accuracy and precision data for the developed method

Level	Predicted Concentration (in mcg/ml)*		Mean % recovery(±S.D)	% Accuracy
	Range	Mean(± S.D)		
LQC	28.38-29.8	29.27±0.494	97.58±1.64	-2.43
MQC	57.97-60.62	59.49±1.02	99.16±1.706	-0.85
HQC	87.56-90.57	88.84±1.144	98.72±1.56	-1.28

*Each value isthe result of nine separate determinations

a. Predicted concentration of candidate drug was calculated bythe linear regression equation.

b. Accuracy is given in % relative error (= 100 × [(predicted concentration – nominal concentration)/nominal concentration)].

3.4.2 Precision

Repeatability was determined using different levels of drug concentrations (the same concentration levels taken in the precision study), prepared from independent stock solution and analyzed (N = 9). Inter-day and intra-day variations and instrument variation were used to determine the intermediate accuracy of the proposed methods. Different levels of drug concentrations were prepared in triplicates three different times in one day and studied for intra-day variation.

The Same protocol was followed for three different days to study inter-day variation (*N* = 9). The relative standard deviation (in %) of the predicted concentrations from the regression equation was taken as precision.

Table 3.3: Results of intermediate precision study

S.No	Concentration (mcg/ml)	Intraday repeatability (n=9)	Interday repeatability (n=9)
1	**30**	**0.87**	**0.982**
2	**60**	**1.146**	**0.66**
3	**90**	**1.176**	**1.02**

3.4.3 Linearity

In phosphate buffered saline, the linearity range was found to be 20-100 μg ml^{-1} at 272 nm with $R^2 = 0.999$

3.4.4 DL and QL

DL and QL were found to be 0.083 μg /ml and 0.25 μg /ml, respectively.

3.4.5 Specificity

The UV spectrum of the drug was not changed in the presence of common excipients in the media selected.

3.5 CONCLUSION

In summary, the proposed method was simple, rapid, precise, accurate and economical and can be used for the routine analysis of TapentadolHCl in bulk, pharmaceutical formulations, and dissolution samples.

3.6 LC-MS-MS BIOANALYTICAL METHOD

3.6.1Objective

The objective of this work was to validate specific LC-MS/MS method for the determination of Tapentadol in rabbit plasma for thePK study of Tapentadol.

3.6.2 Summary

The LC-MS / MS method was performed for the determination of Tapentadol in rabbit plasma. Tapentadol was extracted from the rabbit plasma using the solid phase extraction technique. The final eluent was injected into liquid chromatography equipped with amass detector. The quantification was performed using the peak area ratio method. A 1 / x2 weighting factor was used to determine the concentration of Tapentadol.

Table 3.4: Bio-Analytical Method for Estimation of Tapentadolin Rabbit Plasma

Instrument	**Brand/Model**	**Manufacturer/ Supplier**
Auto sampler	Shimadzu SIL-HTc	Shimadzu Corporation, Japan
Solvent delivery module	LC-10ADvp	Shimadzu Corporation, Japan
Column oven	CTO-10Avp	Shimadzu Corporation, Japan
Degasser	DGU-10A5	Shimadzu Corporation, Japan
MS Detector	TSQ Quantum Ultra	Thermo Electron Corporation, U.K
Vortexer	Spinix/Maxi Mix II	Barnstead International, USA
Centrifuge	Multifuge 3 S-R	Kendro, Germany/Heraeus
	Centrifuge 5810 R	Thermo Electron Corporation, U.K Eppendorf, Germany
Analytical/Micro Balance	Mettler Toledo	Mettler Toledo Laboratory and Weighing Technologies, Switzerland
	Sartorius	Sartorius AG, Germany
Freezer (-70°C)	U725-86/Heraeus	New Brunswick scientific co. inc, England
Freezer (-20°C)	Vestfrost/BFS 345	Blue Star, Denmark
Refrigerator (2-8°C)	RT34M/RT37 MASW1/XTL	Samsung India Electronics Ltd., India
	FF495S	Whirlpool of India Ltd., India
Ultrasonic bath	4020/US-20P	Jeiotech Co. Limited, Korea
Water purification system	Milli-Q Gradiant	Millipore Corporation, USA
Vacuum Pump	Millipore	Millipore Corporation, USA
	Pall	Pall Life Science, India
Positive Pressure Processor	Ezypress 48,	Orochem India Pvt. Ltd., India

Table 3.5: Instrumentation, apparatus and consumables

Apparatus/ Consumables	**Grade/Capacity**	**Manufacturer/ Supplier**
Micropipette	1-10 µl, 2-20µl	Eppendorf AG, Germany / Biohit PLC, Finland
Micro tube	MCT-175, 1.7ml clear	Axygen Scientific, USA
Ria vials	12 X 75 mm PP	Tarsons Products Pvt. Ltd., India
Analytical Column	Beta basic C8, 100 x 4.6 mm, 5µ	Thermo Electron Corporation, UK
SPE Cartridge	Celerity deluxe DVB LP, 30 mg, 1ml	Orochem India Pvt. Ltd., India

3.6.3 Internal Standard

Tramadol was used as internal standard for Tapentadol.

Table 3.6: Internal Standard

Chemicals/Reagents	Grade	Manufacturer/ Supplier
Orthophosphoric acid	Suprapur®	Merck, Germany
Methanol	Gradient grade	Merck , Germany
	HPLC grade	Rankem, India
Formic acid	Suprapur®	Merck, Germany
Acetonitrile	Gradient grade	Merck , Germany
Water	Milli-Q	Millipore, USA

3.6.4 Biological Source

Rabbit blank plasma was purchased from the Preclinical Safety Assessment Department for the preparation of plasma calibration standards and quality control samples. For the verification study of hemolyzed specificity and heparinized plasma were obtained from the Department of Pre-Clinical Safety Assessment. Heparin was used as an anticoagulant.

3.6.5 Type of extraction

Solid phase extraction technique was followed as per mentioned procedure:

Sample Treatment using positive pressure processor and SPE cartridge Celerity deluxe DVB LP was used

25.0 µl of Internal standard spiking solution was transferred to respective micro tube vials except in blank plasma (25.0 µl of diluent was added tosample).

50.0 µl Plasma sample (System Suitability, blank plasma, zero standard, calibration standard, quality control sample and Study samples) was transferred to micro tube/ria vials which containing internal standard using micropipette and vortexed to mix.200.0 µl of 1% v/v Orthophosphoric acid was added in water in the same in microtube vials and vortexed to mix.

Conditioning of SPE Cartridge

1000.0 µl of methanol was loaded to cartridge and pressure was applied.1000.0 µl of Milli-Q water was loaded to cartridge and pressure was applied. To the above plasma,a

mixture was loaded on previously conditioned SPE cartridge and thepressure was applied.

Washing SPE Cartridge

1000.0 µl of Milli-Q water was loaded to cartridge and pressure was applied (1st wash).1000.0 µl of Milli-Q water was loaded to cartridge and pressure was applied (2nd wash).1000.0 µl of 10% v/v Methanol in water was loaded to cartridge and pressure was applied (3rd wash).500.0 µl of elution solution was loaded to SPE cartridge and pressure was applied for elution.

Elution was transferred to 96 deep well collection plate. (1ml/2ml capacity). Above sample was then loaded tothe auto sampler and 10.0 µl was injected.

3.6.7 Linearity Group

The calibration curves were linear from 2.000 ng/ml to 500.000 ng/ml for Tapentadol.

3.6.8 Quantification Parameter

The quantification parameters were performed as per LC Quan software, version - 2.5.6.

Table 3.7: Reference Standards / Working Standards

Reference standard {Analyte}		
Name	:	TapentadolHCl
Batch No.	:	OP-TOP/12/10/007
Retest Date	:	Nov'2015
Name and address of manufacturer	:	Optimus Drugs (P) Limited, India.
Reference standard {Internal standard}		
Name	:	Tramadol Hydrochloride
Batch Number.	:	TRA/WS/001
Retest Date	:	May-2015
Name and address of manufacturer	:	LAVYBENS PHARMA, India.

3.6.9 Preparation of the Calibration Standards and Quality Control Samples

The calibration standards and quality control samples were freshly prepared for the application.

3.6.10 Labeling and Storage

A) Aqueous stock solutions

The stock solutions were labeled to indicate the analyte name, standard identification and date of preparation. These solutions were stored at 2-8°C and at room temperature as per their application.

B) Plasma samples

The freshly prepared samples were labeled to indicate the analyte name, standard identification (calibration standard or quality control sample) and date of spiking. These samples of (respective blank, standard and quality control) were aliquoted and were stored inthe deep freezer (-70^0C/-20^0C).

3.6.11 Calculation of the Sample Concentration

The concentration of the analytes was calculated from the following equation using the linear regression analysis of the plasma calibration standard punctuated with $1 / x_2$ as a weighting factor.$Y = mx + c$, where y is the peak area ratio of the analyte to the internal standard, m is the slope of the calibration curve, x is the analyte concentration and c is the y-axis of the calibration curve

3.6.12 Validation and Characteristics of the Method

A) Chromatography

Mobile phase consisted ofAcetonitrile: Methanol: 0.1% v/v formic acid in water (25:25:50, v/v) with a flow rate of 0.6mL/ml using Beta C8,100 x 4.6 mm, 5µmm column maintained at 30^0C, injection volume 10 µl, RT 1.8 min was used for analysis.

The chromatograms representative of the system suitability, the blank plasma, the samples of LLOQ, ULOQ, LQC, MQC, HQC and the calibration curve of Tapentadol, respectively. (Figure 3.6.1. to 3.6.9)is enclosed at the end of Chapter 3, page numbers 52-56.

B) Specificity / Selectivity

Six different batches of normal heparinized plasma were chromatographed and a batch of heparinized hemolyzed plasma was chromatographed and area response to

TapentadolRT was observed in blank plasma <20.00% LLOQ area response and no area response was observed and compared with respect to Tramadol RT in Blank Plasma (Annexure (Table 3.6.1),is enclosed at the end of Chapter 3, page number 57.

C) Sensitivity

The LLOQ was 2.000ng/ml for Tapentadol.

The % CV of Tapentadol at LLOQ was found to be 10.46

The % nominal concentration for LLOQ samples of Tapentadolwasin the range from 84.60 to 109.65 (AnnexureTable No 3.6.2), is enclosed at the end of Chapter 3, page number 58.

D) Carry over Check

The area response to TapentadolRTin blank plasma was observed <20.00% LLOQ area response and no area response was observed to Tramadol RT in blank plasma (Annexure Table No. 3.6.3)is enclosed at the end of Chapter 3, page number 59.

E) Linearity

The linearity of the method was determined by weighted least squares linear regression analysis of standard plots associated with a standard nine point calibration curve. The best-fit calibration curves of the peak area ratio versus the concentration were drawn. The calibration curves of Tapentadol were linear from 2,000ng / ml to 500,000ng / ml with correlation coefficient of $r \geq 0.9954$ (Annexure Table No. 3.6.4 and 3.6.5), is enclosed at the end of Chapter 3, page number 60.

3.6.13 Accuracy

Table 3.8Within-batch or intra-batch Accuracy

S.No	Parameter	Acceptance Criteria	Result
1	Within-batch or intra-batch accuracy (PandA-02) (Sample treatment by Positive Pressure Processor)	Mean % Nominal concentration:	
		For LLOQ: 80.00 - 120.00	90.85
		For LQC: 85.00 - 115.00	104.82
		For MQC: 85.00 - 115.00	99.39
		For HQC: 85.00 - 115.00	95.61
2	Within-batch or intra-batch accuracy (PandA-03) (Sample treatment by Positive Pressure Processor)	Mean % Nominal concentration:	
		For LLOQ: 80.00 - 120.00	85.50
		For LQC: 85.00 - 115.00	101.17
		For MQC: 85.00 - 115.00	100.27

S.No	Parameter	Acceptance Criteria	Result
		For HQC: 85.00 - 115.00	97.57
3	Within-batch or intra-batch accuracy (PandA-04) (Sample treatment by Positive Pressure Processor)	Mean % Nominal concentration:	
		For LLOQ: 80.00 - 120.00	97.45
		For LQC: 85.00 - 115.00	103.58
		For MQC: 85.00 - 115.00	99.40
		For HQC: 85.00 - 115.00	94.17
4	Within-batch or intra-batch accuracy (PandA-05) (Sample treatment by Positive Pressure Processor)	Mean % Nominal concentration:	
		For LLOQ: 80.00 - 120.00	98.30
		For LQC: 85.00 - 115.00	102.47
		For MQC: 85.00 - 115.00	99.02
		For HQC: 85.00 - 115.00	99.70
5	Within-batch or intra-batch accuracy (PandA-06) (Sample treatment by Positive Pressure Processor)	Mean % Nominal concentration:	
		For LLOQ: 80.00 - 120.00	103.65
		For LQC: 85.00 - 115.00	104.87
		For MQC: 85.00 - 115.00	98.90
		For HQC: 85.00 - 115.00	99.03

Table 3.9 Between-batch or inter-batch Accuracy

S.No	Parameter	Acceptance Criteria	Result
1	Between-batch or inter-batch accuracy	Mean % Nominal concentration:	
		For LLOQ : 80.00 - 120.00	95.15
		For LQC : 85.00 - 115.00	103.38
		For MQC : 85.00 - 115.00	99.40
		For HQC : 85.00 - 115.00	97.22

3.6.14 Precision

Table 3.10 Within-batch or intra-batch Precision

S.No	Parameter	Acceptance Criteria	Result
1	Within-batch or intra-batch precision (PandA-02) (Sample treatment by Positive Pressure Processor)	% CV :	
		For LLOQ ≤ 20.00	10.46
		For LQC ≤ 15.00	3.18
		For MQC ≤ 15.00	1.83
		For HQC ≤ 15.00	1.19
2	Within-batch or intra-batch precision (PandA-03) (Sample treatment by Positive Pressure Processor)	% CV :	
		For LLOQ ≤ 20.00	8.83
		For LQC ≤ 15.00	3.23
		For MQC ≤ 15.00	1.99

S.No	Parameter	Acceptance Criteria	Result
		For HQC ≤ 15.00	0.90
3	Within-batch or intra-batch precision (PandA-04) (Sample treatment by Positive Pressure Processor)	% CV :	
		For LLOQ ≤ 20.00	2.77
		For LQC ≤ 15.00	2.25
		For MQC ≤ 15.00	1.88
		For HQC ≤ 15.00	1.62
4	Within-batch or intra-batch precision (PandA-05) (Sample treatment by Positive Pressure Processor)	% CV :	
		For LLOQ ≤ 20.00	6.26
		For LQC ≤ 15.00	1.43
		For MQC ≤ 15.00	1.33
		For HQC ≤ 15.00	1.00
5	Within-batch or intra-batch precision (PandA-06) (Sample treatment by Positive Pressure Processor)	% CV :	
		For LLOQ ≤ 20.00	2.32
		For LQC ≤ 15.00	1.19
		For MQC ≤ 15.00	1.55
		For HQC ≤ 15.00	0.47

Table 3.11 :Between-batch or inter-batch Precision

S.No	Parameter	Acceptance Criteria	Result
1	Between-batch or inter-batch precision	% CV :	
		For LLOQ ≤ 20.00	9.14
		For LQC ≤ 15.00	2.63
		For MQC ≤ 15.00	1.68
		For HQC ≤ 15.00	2.39

3.6.15 Recovery

Table 3.12: Recovery of Tapentadol

S.No	Parameter	Acceptance Criteria	Result		
1	Recovery of Tapentadol	Recovery should be consistent	Mean % Recovery for		
			LQC	:	92.98
			MQC	:	86.52
			HQC	:	92.99
		% CV within the QC level should be ≤ 15.00		Unextracted	Extracted
			LQC :	1.75	2.83
			MQC :	1.49	1.98
			HQC :	1.09	0.90

		% CV across the QC level should be ≤ 20.00	4.11

Table 3.13 : *Dilution Integrity*

S.No	Parameter	Acceptance Criteria	Result
1	Dilution integrity	% Nominal concentration:	
		For ½ of 2HQC: 85.00 - 115.00	93.32
		For ¼ of 2HQC: 85.00 - 115.00	101.10
		% CV:	
		For ½ of 2HQC ≤ 15.00	0.65
		For ¼ of 2HQC ≤ 15.00	4.31

3.7 MATRIX EFFECT AND MATRIX FACTOR

3.7.1 Matrix Effect

In order to ensure the effect of matrix throughout the application of the method, matrix blanks obtained from seven different lots (06 Heparinised and 01 Haemolysed) were spiked with Tapentadol and Tramadol (IS) at LQC and HQC level, Single quality control samples at each level along with the set of calibration standards were analyzed and the % nominal concentration of the samples analyzed range from 94.02 to 105.72 for Tapentadol (Annexure Table No.3.7.1)is enclosed at the end of Chapter 3, page number 63.

3.7.2 Matrix Factor

The quantitative measure of matrix effect as Matrix Factor (MF) was performed at Low Quality Control (LQC) concentration in at least seven different lots ofthe same type of matrix, out of which 06 should be normal heparinized plasma and 01 Haemolysed plasma with heparin. There was no impact/effect of different plasma lots or plasma composition on the method reproducibility with respect to selectivity, precision, and accuracy of results.

The variability in matrix factors (for the seven different lots), as measured by the coefficient of variation (%CV) was 1.40 % for Tapentadol. (Table No.: 1.6.2, page number) The variability in IS normalized matrix factor (for the seven different lots), as

measured by the coefficient of variation (%CV) was 1.47 % for Tapentadol. (Annexure Table No.: 3.7.2 is enclosed at the end of Chapter 3, page number 64.

3.8 STABILITY

3.8.1 Stock solution stability

The stability of the stock solution was determined by comparing the peak area of the freshly prepared solutions with stability samples.

3.8.2 Main stock solution stability of Tapentadol and Tramadol (6 days at 2-8°C)

The major stock solution of Tapentadol and Tramadol was re-prepared and stock aliquots were maintained at 2-8^{0}C for 6 days (stability sample). The aqueous equivalents of the highest calibration standards of Tapentadol and Tramadol were prepared from the stability sample and analyzed. Stability sample and freshly prepared sample were compared to determine% average change over the stability period.

Table 3.14 :Stock solution stability of Tapentadol and Tramadol (6 days at 2-8°c)

S.No	Parameter	Acceptance Criteria	Result		
1	Main stock solution stability of Tapentadol(6 days at 2-8°c)		Comparison samples		Stability samples
		% CV at ULOQ should be ≤ 5.00	0.49		0.16
		% Mean change at ULOQ ± 10.00	-0.12		
2	Main stock solution stability of Tramadol (6 days at 2-8°c)		Comparison samples		Stability samples

		% CV at ULOQ should be ≤ 5.00	0.88		0.94
		% Mean change at ULOQ ± 10.00	-0.09		

3.8.3 Main stock solution stability of Tapentadol and Tramadol (11 hours at room temperature)

The major stock solution of Tapentadol and Tramadol was re-prepared and stock aliquots were kept at room temperature for 11 hours (stability sample). The aqueous equivalents of the highest calibration standards of Tapentadol and Tramadol were prepared from the stability sample and analyzed. Stability sample and freshly prepared sample were compared to determine% average change over the stability period.

Table 3.15: Stock solution stability of Tapentadol and Tramadol (11 hours at room temperature)

S.No	Parameter	Acceptance Criteria	Result		
1	Main stock solution stability of Tapentadol (11 hrs. at room temp.)		Comparison samples		Stability samples
		%CV at ULOQ should be ≤ 5.00	0.49		0.25
		% Mean change at ULOQ ± 10.00	-0.90		
2	Main stock solution stability of Tramadol (11 hrs. at room temp.)		Comparison samples		Stability samples

		%CV at ULOQ should be ≤ 5.00	0.88		0.46
		% Mean change at ULOQ ± 10.00	-1.55		

3.8.4 Spiking stock solution stability of Tapentadol and Tramadol (6 days at 2-8°C)

A stock solution of Tramadol was freshly prepared and stock aliquots were kept from the stock at room temperature for 11 hours (stability sample). The aqueous equivalents of the highest Tramadol calibration standards were prepared from the stability sample and analyzed. Stability sample and freshly prepared sample were compared to determine % average change over the stability period.

Table 3.16 : Spiking stock solution stability of Tapentadol and Tramadol (6 days at 2-8°C)

S.No	Parameter	Acceptance Criteria	Result		
1	Spiking stock solution stability of Tapentadol (6 days at 2-8°c)		Comparison samples		Stability samples
		%CV at ULOQ should be ≤ 5.00	0.49		0.36
		% Mean change at ULOQ ± 10.00	-0.04		
2	Spiking stock solution stability of Tramadol (6 days at 2-8°c)		Comparison samples		Stability samples
		% CV at ULOQ should be ≤ 5.00	0.88		0.43
		% Mean change at ULOQ ± 10.00	-0.28		

3.8.5 Bench top stability

A) Bench top stability of Tapentadol at room temperature for 9 hours

The LQC and HQC samples were scored in rabbit plasma and maintained at room temperature for 9 hours and processed and analyzed along with freshly prepared LQC and HQC samples. Concentrations were calculated to determine the% change in weight during the stability period. It was found that Tapentadol was stable in samples of LQC and HQC for 9 hours at room temperature with mean % change of -0.50 and 0.17 respectively.

Table 3.17 : Bench top stability of Tapentadol at room temperature for 9 hours

S.No	Parameter	Acceptance Criteria	Result
1	Bench top stability of Tapentadol (9 hrs. at room temp.)	% Mean change :	
		For LQC : ± 15.00	-0.50
		For HQC : ± 15.00	0.17

B) Freeze and thaw stability

Freeze and thaw stability of Tapentadol in Rabbit plasma was evaluated after 4th cycle at -70°C and -20°C.Samples were prepared at LQC and HQC levels, divided into aliquots and frozen at -70°C and -20°C. Six samples of each concentration were subjected to four cycles of freezing and thawing (stability samples). These samples were processed after the fourth cycle and analyzed along with newly processed calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine the mean% change after the fourth cycle.Tapentadol was found to be stable at -70°C in LQC and HQC samples after the 4th cycle with a mean percentage change of -2.32 and 1.93 respectively. Tapentadol was found to be stable at -20 ° C in LQC and HQC samples after the 4th cycle with a mean% change of -0.77 and 3.19 respectively.

Table 3.18: Freeze and Thaw stability of Tapentadol

S.No	Parameter	Acceptance Criteria	Result
1	Freeze and Thaw stability of Tapentadol after 4th cycle at -70°C	% Mean change :	
		For LQC : ± 15.00	-2.32
		For HQC: ± 15.00	1.93
2	Freeze and Thaw stability of Tapentadol after 4th cycle at -20°C	% Mean change :	
		For LQC : ± 15.00	-0.77
		For HQC: ± 15.00	3.19

3.9 PROCESS STABILITY

3.9.1 Process stability of Tapentadol at 5°C in auto sampler for 53 hours

LQC and HQC samples were prepared and processed. These processed samples were kept inan auto sampler at 5°C for 53 hours. These samples were analyzed after 53 hours along with freshly prepared LQC and HQC samples. Concentrations were calculated to determine % of mean change over the stability period. It was found that the Tapentadol was stable in the samples of LQC and HQC for 53 hours at 5°C in an automatic sampler with an average change of -2.72 and -0.36 respectively.

Table 3.19 :Process stability of Tapentadol

S.No	Parameter	Acceptance Criteria	Result
1	Process stability of Tapentadol (after 53 hrsin Auto sampler at 5°C).	% Mean change :	
		For LQC : ± 15.00	-2.72
		For HQC : ± 15.00	-0.36

3.9.2 Summary of Rejected Validation Parameters

Summary of rejected validation parameters during method validation are presented with their respective reasons

Table 3.20 : Rejected Validation Parameters

S.No.	LC-MS/MS ID	Validation Parameters	Reason for Rejection
1	BAN/LCMS/089	Linearity -01, PandA-01	QC Samples were not within acceptance criteria.

3.10 CONCLUSION

The results of the validation of the method for Tapentadol are summarized in tables at the end of this chapter. The analytical method was valid for the analysis of Tapentadol with a calibration interval of 2.000 ng/ml to 500.000 ng/ml in rabbit plasma using Tramadol asan internal standard.

FIGURES –Bioanalytical Methods Representative Chromatograms

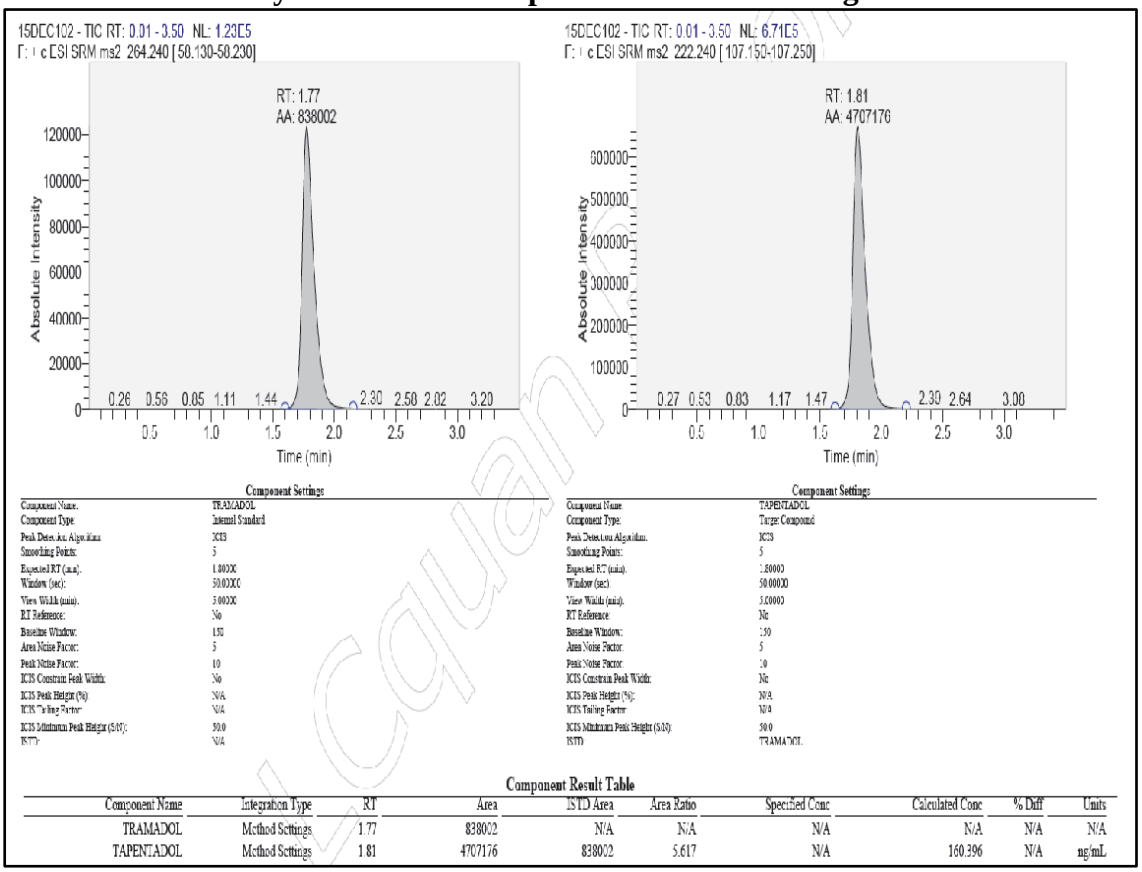


Figure 3.6.1 :Representative chromatogram of system suitability (Chapter 3, Page No. 42**)**

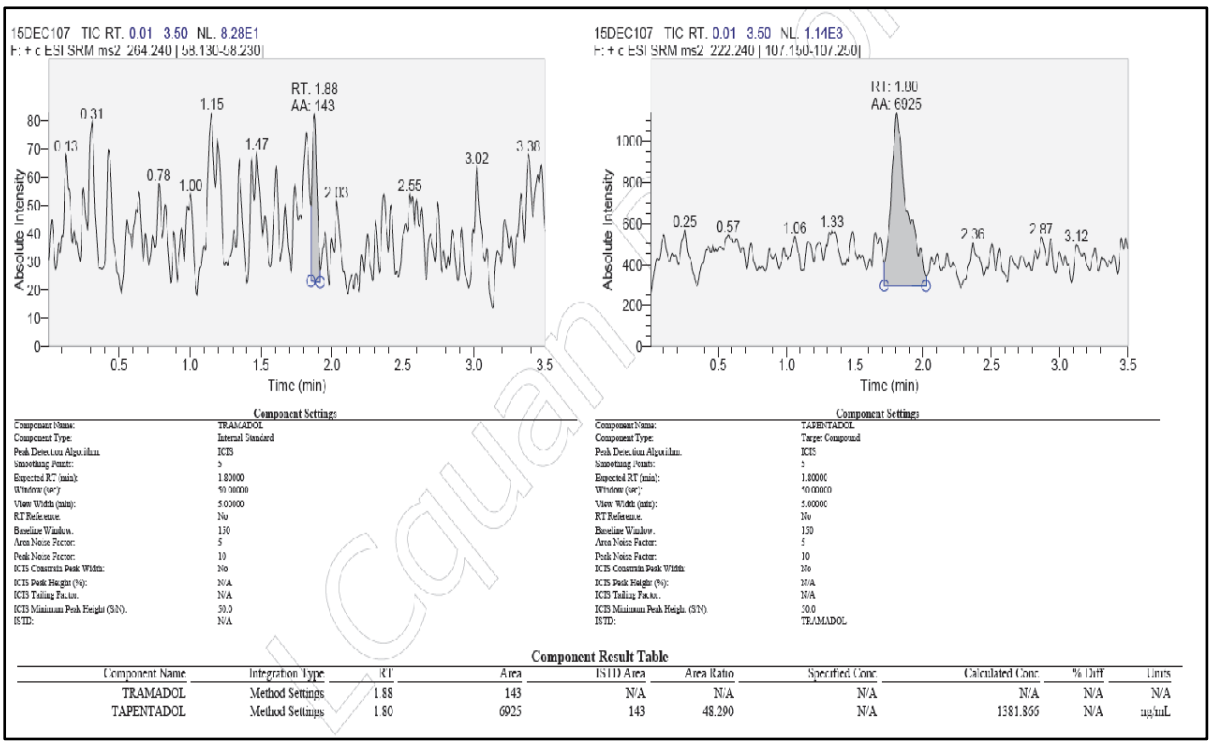


Figure 3.6.2 : Representative chromatogram of blank plasma (Chapter3, Page No.42**)**

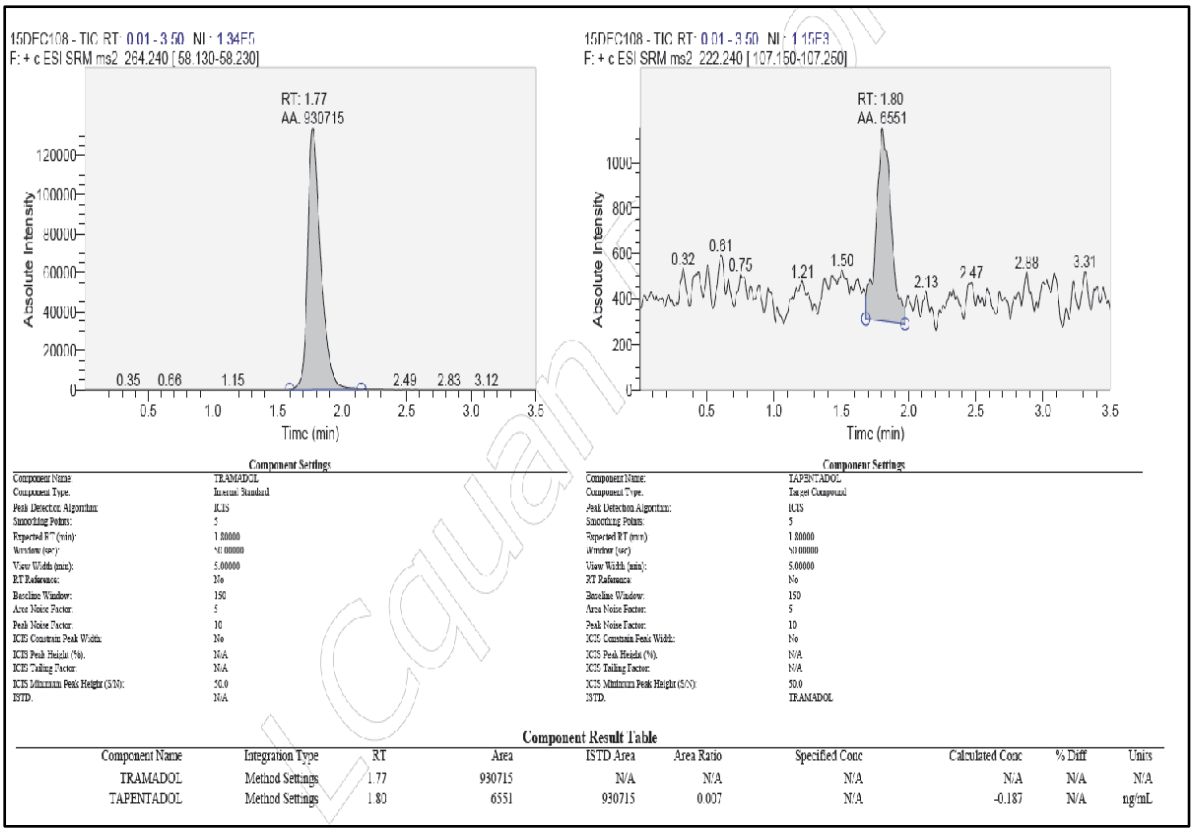


Figure 3.6.3 :Representative chromatogram of zero standard(Chapter 3, Page No. 42**)**

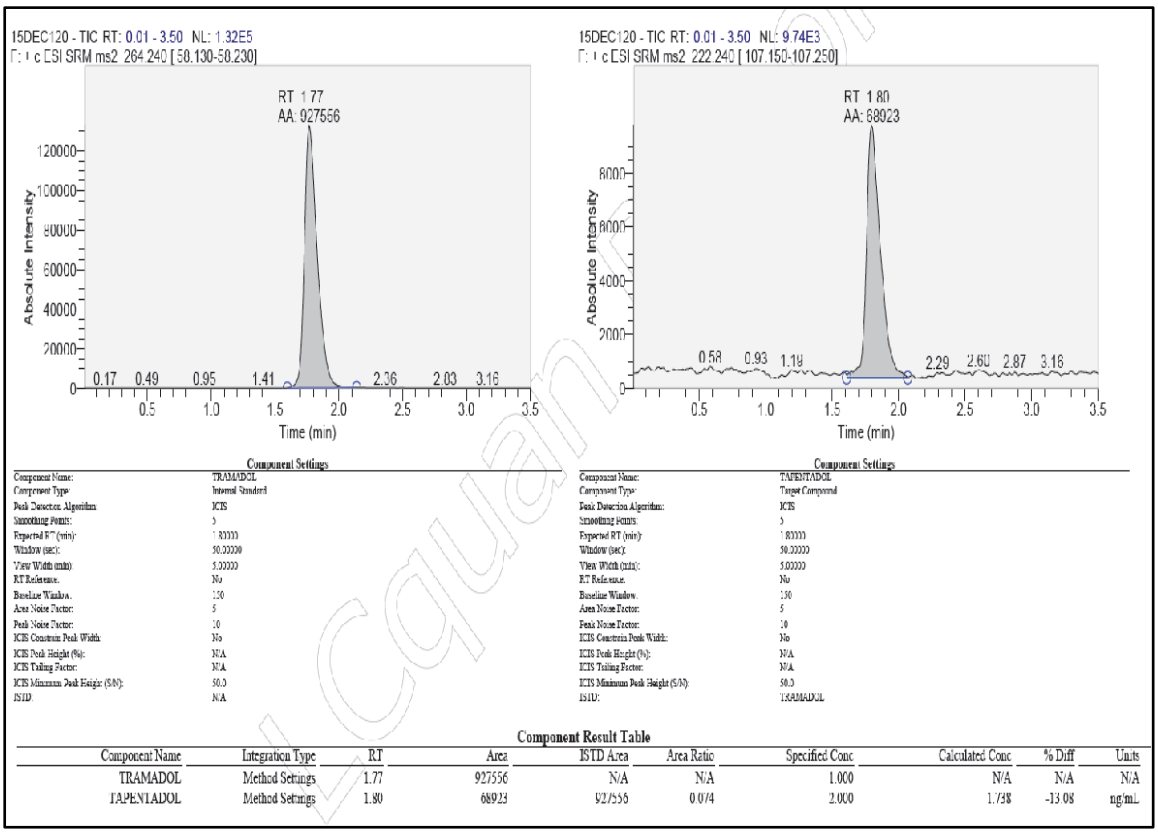


Figure 3.6.4 :Representative chromatogram of LLOQ (Chapter 3, Page No. 42)

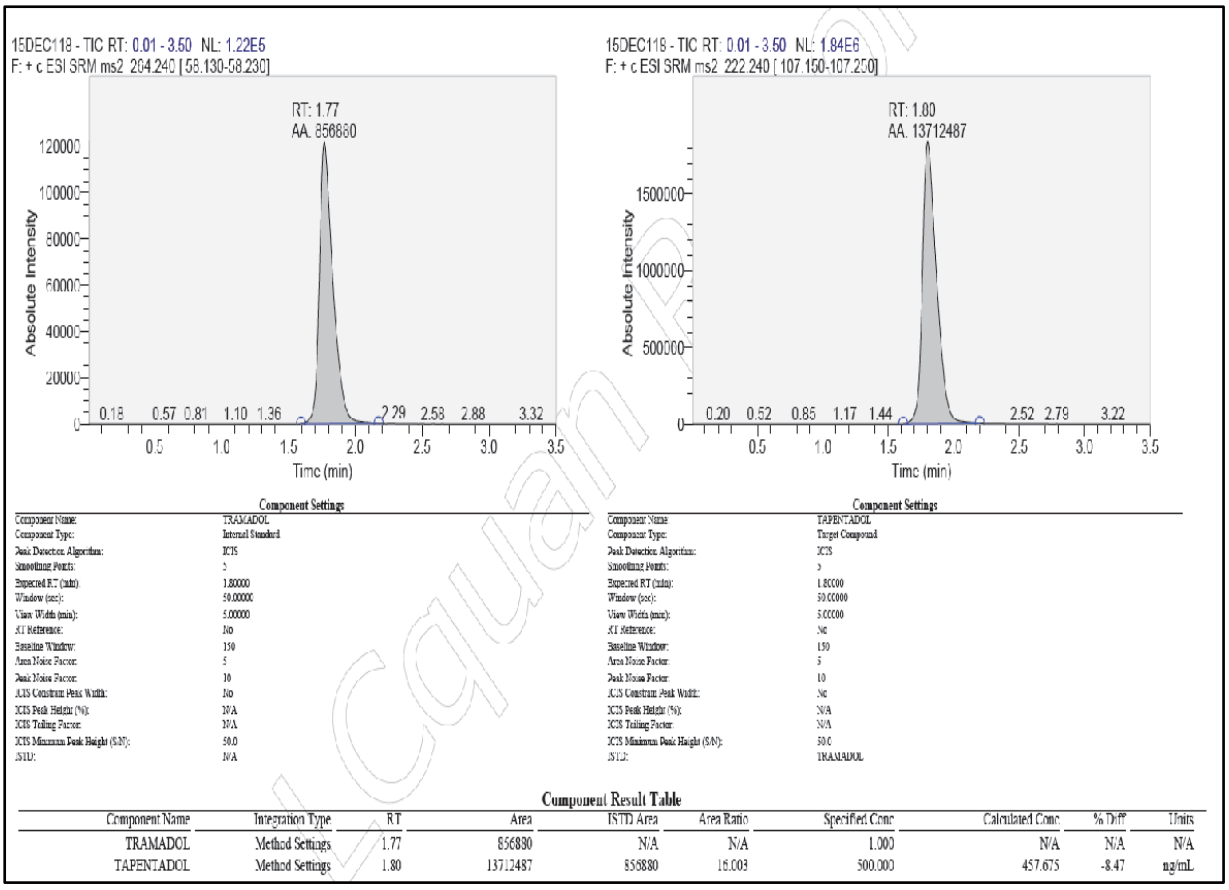


Figure 3.6.5 :Representative chromatogram of ULOQ (Chapter 3, Page No. 42)

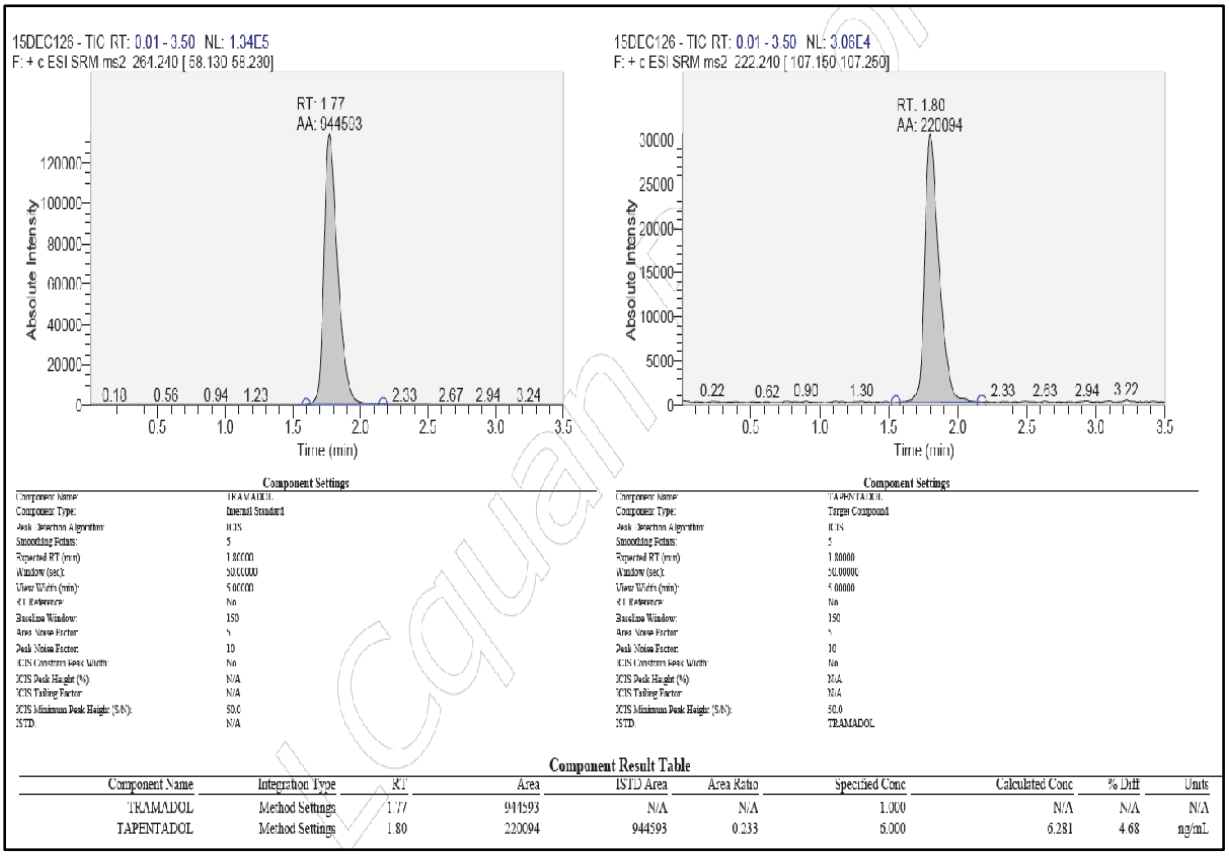
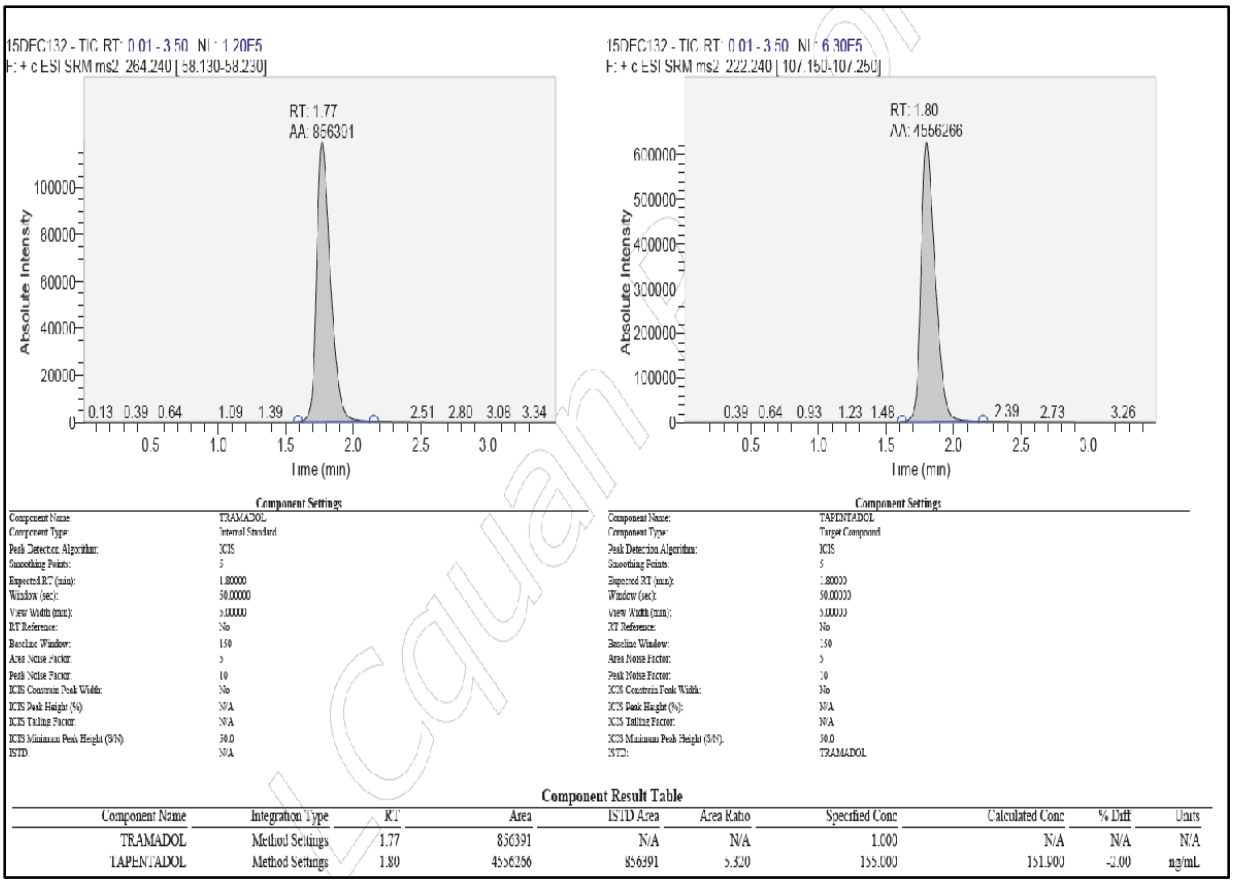
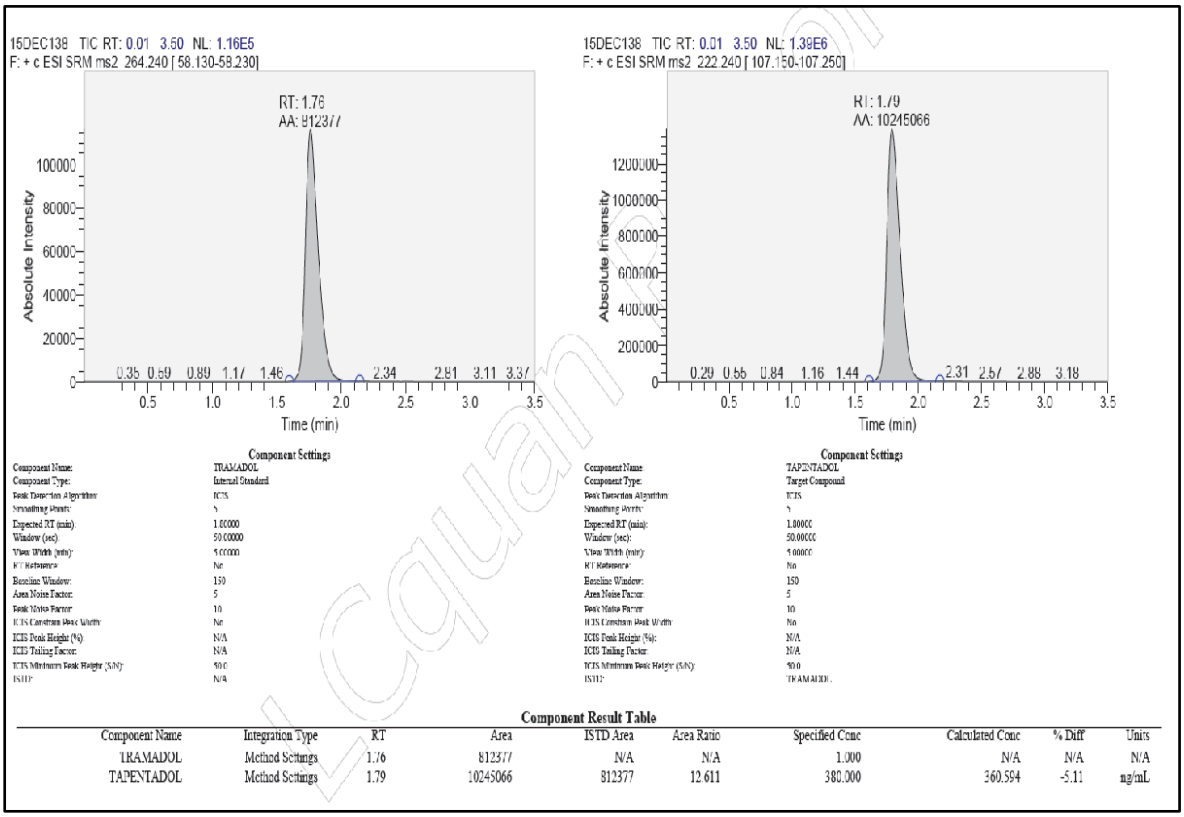


Figure 3.6.6 :Representative chromatogram of LQC (Chapter 3, Page No. 45)



Component Name	Integration Type	RT	Area	ISTD Area	Area Ratio	Specified Conc	Calculated Conc	% Diff	Units
TRAMADOL	Method Settings	1.77	856391	N/A	N/A	1.000	N/A	N/A	N/A
TAPENTADOL	Method Settings	1.80	4566266	856391	5.320	155.000	151.900	-2.00	ng/mL

Figure 3.6.7 : Representative chromatogram of MQC (Chapter 3, Page No. 45**)**



Component Name	Integration Type	RT	Area	ISTD Area	Area Ratio	Specified Conc	Calculated Conc	% Diff	Units
TRAMADOL	Method Settings	1.76	812377	N/A	N/A	1.000	N/A	N/A	N/A
TAPENTADOL	Method Settings	1.79	10245066	812377	12.611	380.000	360.594	-5.11	ng/mL

Figure 3.6.8 : Representative chromatogram of HQC (Chapter 3, Page No. 45)

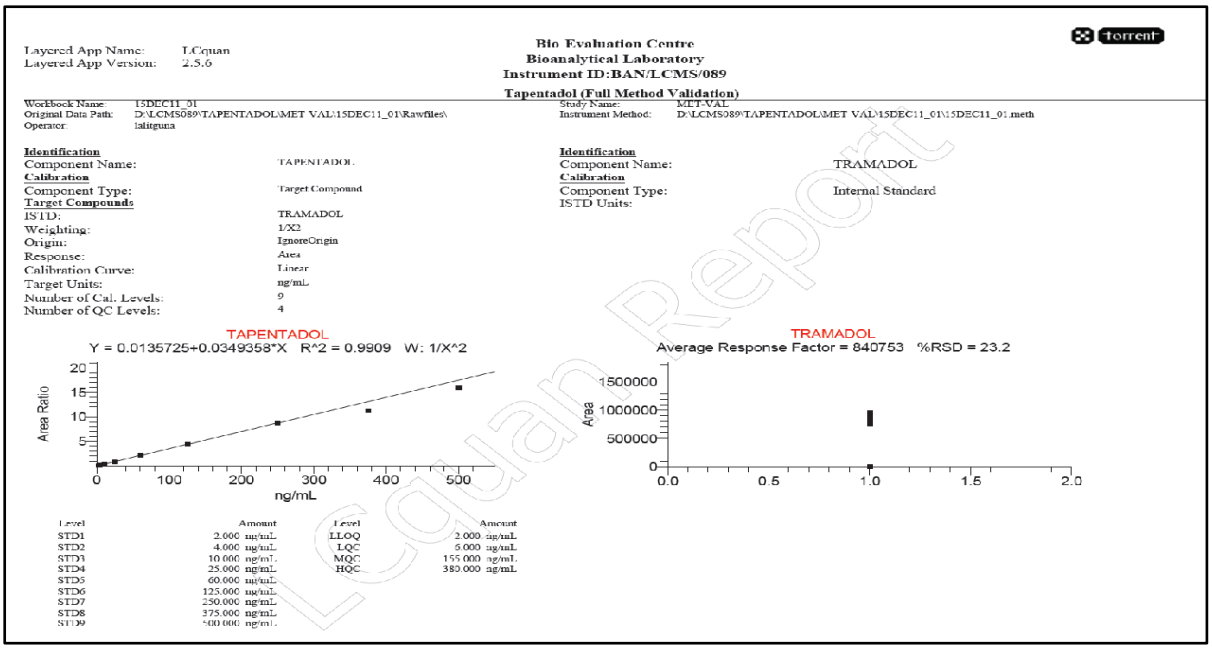


Figure 3.6.9 :Representative calibration curve of Tapentadol(Chapter 3, Page No. 42)

Table No. 3.6.1 (Chapter 3, Page No. 42)

SPECIFICITY AND SELECTIVITY OF BLANK RABBIT PLASMA FOR TAPENTADOL AND TRAMADOL

S. No.	Sample Name	Area		% of area	
		Tapentadol	Tramadol	Tapentadol	Tramadol
1	EXTRACTED BLANK LOT(RBPUP-001-11)	1870	0	3.28	0.00
2	LLOQ LOT(RBPUP-001-11)	57082	778857	NA	NA
3	EXTRACTED BLANK LOT(RBPUP-002-11)	5624	0	9.21	0.00
4	LLOQ LOT(RBPUP-002-11)	61049	754413	NA	NA
5	EXTRACTED BLANK LOT(RBPUP-003-11)	2857	0	4.35	0.00
6	LLOQ LOT(RBPUP-003-11)	65631	822129	NA	NA
7	EXTRACTED BLANK LOT(RBPUP-004-11)	5304	0	9.43	0.00
8	LLOQ LOT(RBPUP-004-11)	56263	708687	NA	NA
9	EXTRACTED BLANK LOT(RBPUP-005-11)	5380	0	8.46	0.00
10	LLOQ LOT(RBPUP-005-11)	63566	800896	NA	NA
11	EXTRACTED BLANK LOT(RBPUP-006-11)	7462	0	10.79	0.00
12	LLOQ LOT(RBPUP-006-11)	69168	845489	NA	NA
13	EXTRACTED BLANK LOT(RBHUP-001-11)	10215	0	15.5	0.00
14	LLOQ LOT(RBHUP-001-11)	65902	820119	NA	NA

NA: Not Applicable
Blank Plasma Lot No with RBPUP Code: Normal Heparinized Plasma
Blank Plasma Lot No with RBHUP Code: Heparinized Haemolyzed Plasma

Table No 3.6.2(Chapter 3, Page No. 42)

SENSITIVITY (LLOQ OF TAPENTADOL)

S.No.	Sample Name	Conc. (ng/ml)	Area		Area Ratio	Calculated	% Nominal conc.
			Tapentadol	Tramadol		Conc. (ng/ml)	
1	LLOQ	2.000	68923	927556	0.074	1.738	86.90
2	LLOQ	2.000	69391	954606	0.073	1.692	84.60
3	LLOQ	2.000	65808	856642	0.077	1.810	90.50
4	LLOQ	2.000	84682	938893	0.090	2.193	109.65
5	LLOQ	2.000	64492	886800	0.073	1.693	84.65
6	LLOQ	2.000	71519	946504	0.076	1.774	88.70
n						6	
Mean						1.817	90.85
SD						0.190	
%CV						10.46	

Table No 3.6.3 (Chapter 3, Page No. 42**)**

CARRY OVER CHECK FOR TAPENTADOL AND TRAMADOL

Sample Name	**Area**		**% Area**	
	Tapentadol	**Tramadol**	**Tapentadol**	**Tramadol**
EXTRACTED LLOQ	68472	832252	NA	NA
EXTRACTED LLOQ	69738	844830	NA	NA
EXTRACTED BLANK	6930	0	10.03	0.00
EXTRACTED ULOQ	20613007	792122	NA	NA
EXTRACTED BLANK	1353	0	1.96	0.00
EXTRACTED ULOQ	20028071	786340	NA	NA
EXTRACTED BLANK	8329	0	12.05	0.00
Mean of LLOQ	**69105**	**838541**		

NA: Not Applicable

Table No. 3.6.4 (Chapter 3, Page No. 42)

SUMMARY OF CALIBRATION CURVE PARAMETERS OF TAPENTADOL

S. No.	LC-MS/MS ID	INITIAL / START		FINAL/ END		SLOPE	INTERCEPT	R
		DATE	TIME	DATE	TIME			
1	BAN/LCMS/089	15/12/15	15:55	15/12/15	19:03	0.0349358	0.0135725	0.9954
2	BAN/LCMS/089	15/12/15	21:59	16/12/15	02:54	0.0320246	0.0175469	0.9973
3	BAN/LCMS/089	16/12/15	15:31	16/12/15	20:23	0.0366057	0.0157141	0.9980
4	BAN/LCMS/089	17/12/15	00:27	17/12/15	03:35	0.0320935	0.0102708	0.9994
5	BAN/LCMS/089	17/12/15	14:08	17/12/15	18:09	0.0325559	0.00635657	0.9991
6	BAN/LCMS/089	17/12/15	20:34	17/12/15	23:55	0.0364908	0.0133735	0.9975
7	BAN/LCMS/089	19/12/15	13:52	19/12/15	17:50	0.0378057	0.0098907	0.9988

Table No 3.6.5 (Chapter 3, Page No. 42)

BACK CALCULATED CONCENTRATION OF CALIBRATION STANDARDS FROM RESPECTIVE CALIBRATION CURVES OF TAPENTADOL

BATCH ID	Conc. (ng/ml)								
	CS-1	**CS-2**	**CS-3**	**CS-4**	**CS-5**	**CS-6**	**CS-7**	**CS-8**	**CS-9**
	2.000	**4.000**	**10.000**	**25.000**	**60.000**	**125.000**	**250.000**	**375.000**	**500.000**
	1.84	4.46	11.04	25.62	61.47	129.54	248.45	324.84	457.67
% Nominal Deviation (% Bias)	-8.05	11.53	10.41	2.51	2.45	3.64	-0.62	-13.37	-8.47
	1.86	4.472	10.65	25.03	60.33	128.48	247.34	341.09	478.08
% Nominal Deviation (% Bias)	-7.20	11.80	6.44	0.10	0.55	2.79	-1.06	-9.04	-4.38
	1.91	4.22	10.81	25.88	62.06	127.060	241.68	349.74	462.73
% Nominal Deviation (% Bias)	-4.70	5.48	8.14	3.52	3.43	1.65	-3.32	-6.73	-7.45
	1.94	4.17	10.41	25.22	60.69	125.56	245.16	368.14	479.78
% Nominal Deviation (% Bias)	-3.00	4.18	4.14	0.88	1.16	0.45	-1.94	-1.83	-4.04
	1.917	4.26	10.49	25.18	60.01	125.05	245.80	369.42	476.89
% Nominal Deviation (% Bias)	-4.15	6.38	4.84	0.68	0.02	0.04	-1.68	-1.49	-4.62
	1.903	4.206	10.895	26.319	62.397	126.933	238.993	350.808	453.866
% Nominal Deviation (% Bias)	-4.85	5.15	8.95	5.28	4.00	1.55	-4.40	-6.45	-9.23
	1.924	4.166	10.802	25.428	60.815	122.277	249.542	360.495	473.832
% Nominal Deviation (% Bias)	-3.80	4.15	8.02	1.71	1.36	-2.18	-0.18	-3.87	-5.23
Mean	1.898	4.278	10.728	25.524	61.112	126.415	245.283	352.079	468.981
Mean % Nominal Deviation	-5.10	6.95	7.28	2.10	1.85	1.13	-1.89	-6.11	-6.20
SD	0.037	0.132	0.225	0.456	0.891	2.397	3.768	15.805	10.655
%CV	1.95	3.09	2.10	1.79	1.46	1.90	1.54	4.49	2.27

Table No. 3.7.1 (Chapter 3, Page No. 45)

MATRIX EFFECT FOR TAPENTADOL

S. No.	File Name	Sample ID	Nominal	Area		Area Ratio	Calculated	% Nominal
			Conc. (ng/ml)	Tapentadol	Tramadol		Conc. (ng/ml)	Conc.
1	17DEC220	LQC(RBPUP-001-11)	6.000	247884	1031539	0.240	6.219	103.65
2	17DEC221	LQC(RBPUP-002-11)	6.000	245605	1027717	0.239	6.183	103.05
3	17DEC222	LQC(RBPUP-003-11)	6.000	251646	1047602	0.240	6.216	103.60
4	17DEC223	LQC(RBPUP-004-11)	6.000	245058	1026544	0.239	6.175	102.92
5	17DEC224	LQC(RBPUP-005-11)	6.000	241155	1012834	0.238	6.158	102.63
6	17DEC225	LQC(RBPUP-006-11)	6.000	252698	1036915	0.244	6.312	105.20
7	17DEC226	LQC(RBHUP-001-11)	6.000	250916	1024892	0.245	6.343	105.72
8	17DEC227	HQC(RBPUP-001-11)	380.000	12858501	972871	13.217	361.836	95.22
9	17DEC228	HQC(RBPUP-002-11)	380.000	12849529	984547	13.051	357.291	94.02
10	17DEC229	HQC(RBPUP-003-11)	380.000	12853614	952420	13.496	369.473	97.23
11	17DEC230	HQC(RBPUP-004-11)	380.000	12861126	962959	13.356	365.639	96.22
12	17DEC231	HQC(RBPUP-005-11)	380.000	12771540	973892	13.114	359.010	94.48
13	17DEC232	HQC(RBPUP-006-11)	380.000	12733647	949322	13.413	367.217	96.64
14	17DEC233	HQC(RBHUP-001-11)	380.000	12678398	949274	13.356	365.640	96.22

Blank Plasma Lot No with RBPUP Code: Normal Heparinized Plasma

Blank Plasma Lot No with RBHUP Code: Heparinized Haemolyzed Plasma

MATRIX FACTOR FOR TAPENTADOLTable No.3.7.2 (Chapter 3, Page No. 46)

S.No.	USED MATRIX LOT ID	ANALYTE PEAK RESPONSE AT LQC CONC. IN				MATRIX FACTOR (MF)
		FILE NAME	PRESENCE OF MATRIX ION: EXTERNALLY SPIKED SAMPLE	FILE NAME	ABSENCE OF MATRIX ION: AQUEOUS SAMPLE	
1	RBPUP-001-11	17DEC234	242343	17DEC241	237015	1.044
2	RBPUP-002-11	17DEC235	234932	17DEC242	226763	1.012
3	RBPUP-003-11	17DEC236	237263	17DEC243	226222	1.022
4	RBPUP-004-11	17DEC237	236927	17DEC244	235581	1.021
5	RBPUP-005-11	17DEC238	232129	17DEC245	233433	1.000
6	RBPUP-006-11	17DEC239	239877	17DEC246	233454	1.034
7	RBHUP-001-11	17DEC240	236342	NA	NA	1.018
MEAN					232078	1.022
SD						0.0143
% CV						1.40

Blank Plasma Lot No with RBPUP Code: Normal Heparinized Plasma

Blank Plasma Lot No with RBHUP Code: Heparinized Haemolyzed Plasma

NORMALIZED MATRIX FACTOR FOR TAPENTADOL

Table No.3.7.3 (Chapter 3, Page No. 53)

S.No.	USED MATRIX LOT ID	ANALYTE PEAK TO INTERNAL STANDARD (IS) PEAK RESPONSE RATIO AT LQC CONC. IN				IS NORMALIZED MATRIX FACTOR
		FILE NAME	PRESENCE OF MATRIX ION: EXTERNALLY SPIKED SAMPLE	FILE NAME	ABSENCE OF MATRIX ION: AQUEOUS SAMPLE	
1	RBPUP-001-11	17DEC234	0.258	17DEC241	0.247	1.053
2	RBPUP-002-11	17DEC235	0.251	17DEC242	0.245	1.024
3	RBPUP-003-11	17DEC236	0.253	17DEC243	0.243	1.033
4	RBPUP-004-11	17DEC237	0.252	17DEC244	0.245	1.029
5	RBPUP-005-11	17DEC238	0.248	17DEC245	0.248	1.012
6	RBPUP-006-11	17DEC239	0.248	17DEC246	0.243	1.012
7	RBHUP-001-11	17DEC240	0.248	NA	NA	1.012
MEAN					0.245	1.025
SD						0.0151
% CV						1.47

Blank Plasma Lot No with RAPUP Code: Normal Heparinized Plasma

Blank Plasma Lot No with RAHUP Code: Heparinized Haemolyzed Plasma

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