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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DULOXETINE HYDROCHLORIDE IN HUMAN PLASMA USING LC-MS/MS

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ABSTRACT

A simple, sensitive, economical and validated method is developed using LC-MS/MS with electro spray ionization for the quantification of Duloxetine in human plasma. Following protein-precipitation extraction, Separation of analyte and internal standard (Telmisartan) was performed on X-Bridge (4.6 x 50 mm, 3.5µm) column using a gradient elution mode were run in positive mode using Telmisartan as IS with mobile phase composition of 100% methanol and 0.1% formic acid on a reverse phase column and analyzed by MS /MS (API 4000) in the multiple reaction monitoring mode using the respective [M+H] + Ions, m/z 297.90 to 154.1 for Duloxetine and m/z 515.10 to 276.30 for IS. The assay exhibited a linear dynamic range of 0.5 to 200 ng/ml for Duloxetine in human plasma. The lower limit of quantification was 0.345 ng/ml with the relative standard deviation between 1.19% to 13.12 (n=6). Acceptable precision and accuracy were obtained for concentrations over the standard curve range. No significant degradation was observed for Duloxetine in human plasma when stored at room temperature (4h), when subjected to Freeze and thaw cycles (3 cycles). The overall absolute recovery is 73 % to 100%. No matrix suppression was found in the method. Because of less run time of 1.3 minutes per sample.

KEYWORDS

Duloxetine, Telmisartan, Mass Spectroscopy and Internal standard

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INTRODUCTION

Duloxetine Hcl (DLX) is chemically, 2(+)-(*S*) -*N* - methyl - (gamma) - (1-naphthyloxy) - 2 thiophenepropylamine hydrochloride[1]. Duloxetine hydro chloride is a newer selective serotonin and nor epinephrine reuptake inhibitor (SSNRI) used for major depressive disorders[2-3]. The empirical formula is $C_{18}H_{19}NOS$. Hcl and having a molecular weight of 333.88. It is used for the treatment of naturopathic

pain associated with peripheral neuropathy especially diabetic polyneuropathy for which it is first-line and as an add-on treatment in stress urinary incontinence instead of surgery[4-5], also indicated for the management of fibromyalgia[6-7]. It restores the balance of neurotransmitters in the brain like serotonin and norepinehrine[8]. Moreover it is also being used in the treatment of peripheral neuropathy caused by certain anti-cancer drugs[9]. In the references few bio analytical methods are reported for the determination of Duloxetine in human plasma by LC-MS/MS[10], HPLC analysis of Duloxetine in human plasma with SPE[11], capillary electrophoresis with laser-induced fluorescence detection[12], in blood using HPLC with spectrometric detection and column switching[13] and LC-MS (SIM mode)[14].

MATERIAL AND METHODS

Instruments

Electronic Balance- Sartorius Ag, Analytical HPLC- Shimadzu, Auto sampler Pal Hts-xt, Micro pipettes (0.2-2 µl, 2-20µl, 10-100 µl, 20-200 µl, 100-1000 µl) Eppendorf Research and Gilson, Sonicator- Power Sonic 505, pH meter- Mettler Toledo, Freezer(-86^{0}C)- Sanyo, Refrigerator- Haier Pharmaceutical Refrigerator, Centrifuge, max.4000rpm (for 96-well plates)- Megafuge, Heraeus, Centrifuge (max.13000 rpm)- Bio Fuge, fresco. Mass spectrum was measured by API 4000 LC/MS/MS Systems, Applied Bio systems.

Chemicals

A reference standard of Duloxetine was procured from Medrich Laboratories Ltd. Telmisartan and Dimethyl Sulfoxide was procured from Sigma-Aldrich. Methanol, Acetonitrile, glacial acetic acid, 2-propanol and formic acid was of HPLC Grade purchased from Merck Pvt. Ltd, Mumbai. Analytical-grade Ammonium formate and Ammonium acetate was purchased from Merck specialties (Mumbai, India). Sodium fluoride was purchased from SD. Fine-Chem Limited. Water used for the LC-MS/MS analysis was prepared using a Milli Q water purification system procured from Millipore (Bangalore, India).

Method Development/optimization of MS/MS method

Analytes and internal standards were manually infused separately at 10µL/min using the Harvard syringe apparatus. Detection was performed using an API 4000 triple quadruple mass spectrometer (Applied Bio systems/MDS Sciex). Optimum compound dependent parameters such as declustering potential, Entrance Potential, Collision energy, Collision cell exit potential were tuned for mass spec followed by the identification of precursor mass and corresponding fragment ions. Source/gas parameters were optimized through FIA mode. This process was repeated for each of the individual drugs tested and also for internal standards. See Table No.1 for the final tuning parameters.

Precursor ion scans (Q1)

A single chemotherapeutic drug agent was made up in mobile phase at a concentration of 1µg/ml (very concentrated for MS analysis so that the precursor (parent) mass ion could be easily established) and this sample was then infused directly to the mass spectrometer where a TIC scan was run in order to establish the predominant precursor ion. The mass range was set so that it bracketed the molecular weight of the compound under analysis. The source parameters and compound parameters were optimized in such a way to get more intense peak. Once a large peak (highest intensity) was seen at the expected m/z value (protonated, sodiated or potassiated adduct etc.), this was deemed to be the precursor ion. The parameters such as declustering potential and entrance potential were optimized by varying the voltages to get highest intense peak.

Product ion scans (Q_3)

A product ion scan was carried out in order to obtain the product (daughter) ions of the selected precursor ion. The product ion scan test worked by firstly isolating and then fragmenting the chosen precursor ion with varying collision energy values (0-80). The product ions were determined by analyzing the

fragments produced on collision and again determining which were predominant and specific for the precursor ion under examination.

Optimizing collision energy conditions for product ions

Repeated multiple reactions monitoring (MRM) scans which detected the MS/MS transitions were carried out in order to obtain the optimum collision energy for fragmentation of a particular drug to a particular product (daughter) ion. Unlike the product ion scan protocol, in this case collision energy was raised in increments of 5V (from 0 to 5 to 10 etc) so that more accurate collision energy could be established. The collision energy that gave the most intense peak for the chosen product (daughter) ion was deemed to be the optimum value. In this MRM mode collision energy and collision cell exit potential were optimized.

Stock solutions preparation

Primary stock solution of Duloxetine hydrochloride were prepared at 1 mg/ml by weighing 1.916 mg of drug accurately and dissolved in 2.16 ml of methanol in 1.5 ml eppendorf tubes. They were properly vortexed and sonicated for few minutes to dissolve properly. This was split into 200 µL sub aliquots in 0.5 mL microfuge tubes and stored at -20^0C.

Internal standard

Primary stock solution at 2.5 mg/ml was prepared for Telmisartan. From this, working stock solution at 200 ng/ml was prepared by adding 4 µl of each concentrated stock solution to 50 ml of Methanol in 50 ml eppendorf tubes and stored at 2-8^{0}C.

Mobile phases

5M Ammonium formate - Ammonium formate was accurately weighed (630.6mg) and added to 800mL of milli-Q water. After proper mixing volume was adjusted to 1L and then sonicated.100% Acetonitrile. 0.1% formic acid in water – 1ml of formic acid was added to 1L of milli-Q water, mixed properly and sonicated.0.1% formic acid in acetonitrile - 1ml of formic acid was added to 1L of acetonitrile, mixed properly and sonicated.100% Methanol.

Extraction solvent

Methanol containing internal standards (Telmisartan at 200ng/ml concentration).

Mass spectrometry optimization (infusion) solution preparation

100 ng/ml of tuning solutions for each analyte and internal standard were prepared separately by adding 0.2 µl of 2 mg/ml concentrated stock solution to 4 ml of methanol: water (50:50) in 5 ml centrifuge tubes and vortexed for few minutes.

Plasma preparation

The blood was treated with 2% w/v anti-coagulant (K_2EDTA) to prevent the blood from clotting and was mixed and centrifuged at 13000 rpm to separate the plasma. The supernatant plasma is separated into 15 ml centrifuge tubes and stored at -80^{0}C. To this for each ml of plasma 10µl of 50% w/v of sodium fluoride (esterase inhibitor) was added before sample processing step.

Quantitative method for drug analysis in human plasma

Preparation of calibration standards and quality control samples

The calibration standard range was chosen based on the literature reported human plasma concentrations ($C_{max)}$ of each drug. Initially calibration standards were prepared in Methanol: water (50:50) as shown in the below table. Later by using these working calibration standards, plasma (matrix) standards were prepared.6.5µl of Duloxetine hydrochloride was added to 994.00 µl of diluent Methanol water (50:50) for preparing neat solution ULOQ concentration level (SS-09) Later serial dilution was performed till LLOQ level (SS-01). Similarly for 6µl added to make HQC, Later serial dilution was performed till LQC level. These volumes were spiked separately from 1mg/ml stock solutions of each drug into common main stock to get linearity ranges of 0.5-200ng/ml, for Duloxetine hydrochloride respectively (gives cassette main stock). For preparation of matrix solution calibration

standards 2 µl of the cassette mixture (from aqueous linearity) was spiked to 48 µl of plasma and vortexed. Samples were precipitated by adding 150 µl of Telmisartan containing internal standard.

Sample preparation (extraction) procedure

48µl of clean reference plasma (blank plasma which is previously tested for any interference) was spiked into 1.5ml centrifuge tubes for each standard (total of 9) and quality control (low, medium and high concentrations). 2µl of each standard and quality control working stock solution was added separately to the plasma spiked to get 25 times dilution. The centrifuge tubes (eppendorf) were capped and vortexed for a short time. 150µl of Telmisartan containing internal standard was added for each tube and vortexed at 1200rpm for 5 min. using mix-mate. Then they were centrifuged at 13000rpm for 10min at 4^{o}C. After centrifugation 150µl the supernatant was spiked and transferred to a 96-well plate. To this 150µl of milli-Q water was added for each sample well containing standards and quality controls and vortexed for 5min.The samples are ready for injection.

Validation procedure

Precision-Accuracy batch assessment samples

Intra- and inter-day precision and accuracy were performed from three batches, each containing the calibration curve in singlet, specificity samples with or without internal standard (prepared from unspiked, pooled K_2EDTA human plasma), and six replicates of each quality control sample. Specificity (six individual lots) samples were also estimated to find out matrix selectivity for sample processing.

Stability assessment samples

Stability assessment standards (freeze thaw, bench-top, and auto-sampler stability) were prepared using human plasma K_2EDTA at low, medium and high quality control level. The analyte was spiked into the plasma using the dilute stock solution. After spiking, these samples were frozen at -80^{0}C. This differentiated them from calibration standards. They are referred to throughout as spiked frozen plasma standards.

Recovery assessment samples

The recovery of protein precipitation extraction procedure was also evaluated. Extracts of the low, medium and high QC levels were processed and compared with injection of neat standards at the same level concentrations. The total recovery is reported as the ratio of the peak area in the extracted sample to the peak area in the neat standard.

Development/optimization of LC-method

The HPLC-MS/MS method was optimized to identify and quantify the analyte. Firstly, the method was required to adequately separate the target analyte in one LC-MS/MS run. The method conditions including HPLC, ESI and MS parameters were optimized for these target analyte. The identification of target analyte was done using the chromatographic retention time (R_t), the MS of target ions and the fragmentation pattern. Good separation was a key in the method's success. Greater chromatographic resolution allowed for improved signal intensities of these target analyte because enough dwell time was spent on each transition without signal degradation.

HPLC-MS/MS conditions were optimized as follows: 1) Selection of HPLC columns, 2) Selection and characteristics of mobile phase, 3) Development/optimization of multiple reaction monitoring (MRM) transitions for target analyte, 4) Optimization of LC-parameters such as retention time, mobile phase flow rate, gradient or isocratic elution time program, injection volume etc.

The chromatographic trail runs were performed using a medium concentration to get an optimum separation of analytes and intensity for simultaneous analysis. The chromatographic trails performed were mentioned below.

Optimization of Chromatogram for Duloxetine Hydrochloride for Trial-1

Trail-1

Column: synergi 4µ Polar-RP 80A (c18), 30 x 2mm.
Mobile Phase : A – 5mM Ammonium formate
B – 0.1% formic acid in Acetonitrile

Flow rate : 0.8 ml/min
Injection volume : 20µl.

Observation

It has been observed that chromatography was not good as peak shape was bad (peak splitting observed for Duloxetine mobile phase has been optimized.

Optimization of Chromatogram for Duloxetine Hydrochloride for Trial-2

Trail-2

Column: synergi 4µ Polar-RP 80A (c18), 30 x 2mm.
Mobile Phase : A – 5mM Ammonium formate
B – 100% Acetonitrile
Flow rate : 0.8 ml/min
Injection volume : 20µl.

Observation

Mobile phase B was changed and dilution was made for supernatant after extraction. Fronting peak splitting was observed. It has been observed that Duloxetine needs neutral pH for elution.

Optimization of Chromatogram for Duloxetine Hydrochloride for Trial-3

Trail-3

Column: synergi 4µ Polar-RP 80A (c18), 30 x 2mm.
Mobile Phase : A – 5mM Ammonium formate
B – 100% Acetonitrile
Flow rate : 0.8 ml/min
Injection volume : 20µl.

Observation

Still bad chromatograms were observed with broad peaks.

Final Optimization of Chromatogram for Duloxetine Hydrochloride

Column: X-BRIDGE (c18), 4.6 x 50mm, 3.5 µ
Mobile Phase : A – 0.1%Formic acid in water
B – 100% Methanol
Flow rate : 1.0 ml/min.

Observation

Good chromatogram was observed for all analytes after changing the flow rat.

RESULTS AND DISCUSSION

Optimization of MS/MS detection conditions and Parameters

Using infusion of each analyte at 100ng/ml concentration the optimum multi-reaction monitoring (MRM) settings were determined. Table No.3 presents the MRM transitions and optimum compound dependent parameters identified for each analyte the optimized MS-parameters are mentioned in Table No.3.

Precursor ion determination

The precursor ion, also called the parent ion, is “an electrically charged molecular moiety which may dissociate to form fragments. These fragments may be charged or neutral moieties. Charged fragments can be detected, forming the basis of tandem mass spectrometry, and used to further identify the analyte.

Product ion determination

The product ions are the ions detected by the detector in the third quad of the mass spec., as a result of the fragmentation of preselected precursor ions in the collision cells. By exposing the precursor ion to increasing collision energy, fragmentation occurs and the product ions can be determined as the MS2 detector scans.

MRM transition optimization

MRM (multi-reaction monitoring) can track many precursor ions and their multiple product ions through the MS to provide spectra that are quantifiable. Each transition from precursor ion to product ion can have different collision energies. By optimizing the MRM transition, the collision energy for each transition was optimized.

Optimization of LC conditions for Duloxetine hydrochloride

Shimadzu LC method parameters:
Pump-A model : LC-20A
Pump-B model : LC-20A

Pump mode : Binary flow
Total flow : 1.5 ml/min
Column oven Temperature : 40^{o}c
CTC PAL-xt Auto sampler method parameters:
Loop volume-1(µl): 100
Loop volume-2(µl): 100
Injection volume : 10.001µl
Eluent : Eluent-A: 0.1% Formic acid in water
Eluent-B : Methanol 100%
The eluent and the gradient above that we used gives the best response for these compounds in combination with the lowest carryover (we reduced the carryover to the noise level) after the injection of the highest concentration of standards.

Selection of HPLC column

Based on previous studies reversed phase C-18 columns were the most frequently used HPLC column for these target analytes. Initially, conventional silica based C-8 analytical columns (i.e., C_8, X-bridge) were tested and demonstrated poor separation and bad peak shape. Various factors including the composition of the mobile phase and pH levels were also examined; however, no significant improvement in the chromatographic separation was obtained primarily due to their similar physicochemical properties. Synergi 4µ Polar-RP 80A (30×2mm) demonstrated good resolution but showed asymmetrical peaks. Finally C_{18} X-Bridge (4.6×50mm) 3.5µ showed substantially good resolution, symmetrical peaks and less co-elution between target compounds due to its effectiveness in polarity and aromatic selectivity.

Mobile phase optimization

The interaction of mobile phases and LC gradients were optimized to improve LC separation and detection sensitivity for the LC-MS method. Initially Acetonitrile is used as eluent for separation because it promoted π-π interactions between the aromatic rings, but showed poor elution property. Addition of 0.1 % formic acid in Water and 100% methanol showed good resolution.

Validation

The second stage in development of a bioanalytical method is its validation. This is crucial prior to sample quantitation using the method as it establishes the robustness of a method, ensuring that sample analysis will result in precise, accurate results for the amount of analyte in the analytical samples. The purpose of this bioanalytical method validation was to determine whether the method developed would satisfy the requirements of industry guidelines as set out by the FDA. If the method failed to comply with these guidelines, it may be deemed unsuitable and subsequent sample analysis not appropriate for regulatory submission for NCEs. In order for concentration determination of target analytes in human plasma to be possible, there needs to be proof that the method is appropriate for this purpose. This begins with the construction of the calibration line. A calibration line formed from of the line of best fit for the quantitation standards in the range 0.5-200ng/ml, for Duloxetine hydrochloride, was prepared in human plasma. These standards were extracted in the presence of Telmisartan internal standard and analysed by LC-MS/MS using their MRM transitions. The peak areas ratio for analyte to internal standard from the extracted ion chromatograms (XIC) was calculated. Human plasma without analytes, and solutions containing analytes without human plasma were also analysed. The purpose of these analyses was to test if analytes were stable at room temperature for 24 hours in plasma.

Construction of Calibration Curve

In order to determine the linearity of response, five replicate sets of quantitation standards, each set prepared on a different day but using the same plasma and dilute stock solution were prepared, each containing 9 different concentration levels (calibration standards of analytes) in human plasma. This will be referred to as inter and intra-day data, and will show that the assay is robust and reproducible on different days. The aim of the

experiment was to assess the lowest level which could be quantified by LC-MS/MS.

The peak area for each analyte was determined from the extracted ion chromatogram and divided by the peak area of the internal standard obtained in the same way. This was plotted against the nominal concentration of analyte. The simplest appropriate form of regression was chosen as required by the FDA. In this case it was linear 1/x or $1/x^2$ where x was the concentration. This gave the calibration curve a stronger weighting towards the lower end of the calibration curve.

Carryover

Within each batch, the specific sample without internal standard is injected immediately after the highest standard to verify that carryover is less than 20% of the LLOQ on a peak area basis.

Method selectivity (matrix selectivity)

The MRM transitions of each analyte were shown to be selective and specific when used to analyze six individual lots of unspiked K_2EDTA human plasma. The matrix selectivity results were shown in Table No.4.

Linearity

The calibration curve for analytes was generated using a 1^x linear regression or quadratic regression and performed by the Analyst 1.5 software. The back calculated intra-batch data for standard curves is presented in tables mentioned below. Standards with a back-calculated accuracy outside the range of 85-115% of the nominal concentration were excluded from the regression statistics. The correlation coefficients for all calibration curves were more than 0.99 results were shown in Table No.5.

Accuracy and Precision

Intra-batch (n=6/batch, 3 days) and inter-batch (n=18) accuracy and precision for each analyte are shown below. The results show acceptable accuracies, especially when taking into account the multistep sample preparation procedure, with most between 90-110% of the nominal concentration. The coefficients of variation for each analyte, both intra- and inter-day, are also well below the nominal criteria of less than 15%. The results were shown in Table No.6.

Recovery

The recovery for analytes from K_2EDTA human plasma from the LQC, MQC and HQC level was determined by comparing with their respective aqueous quality 76% (n=6) respectively. The results were shown in Table No.7 and 8.

Stability

Freeze-thaw stability

Freeze-thaw stability of low, mid and high level quality control samples was validated for three cycles of freezing and thawing. The calculated values for the freeze-thaw stability samples were within the precision and accuracy range determined by the inter-day QC samples, leading to the conclusion that the analytes showed no instability due to freezing and thawing over the three cycles.

Auto-sampler stability

Auto-sampler stability of K_2EDTA human plasma quality control samples was validated by keeping the samples for 24hrs duration in auto-sampler (temperature condition at 4^0C) and then compared with fresh samples. The calculated values for auto-sampler stability(n=6 at low, mid and high QC levels) were within the precision and accuracy range determined by the inter-day QC samples, leading to the conclusion that the analytes showed no instability due to auto-sampler storage of the samples throughout the run time.

Bench-top stability

Bench-top stability of K_2EDTA human plasma quality control samples was validated by keeping the samples for 4hrs at the room temperature where the sample processing is carried out and then they compared were with fresh samples. The calculated values for bench-top stability(n=6 at low, mid and high QC levels) were within the precision and accuracy range determined by the inter-day QC samples, leading to the conclusion that the analytes showed no instability

due to auto-sampler storage of the samples throughout the run time.

DISCUSSION

The quantitative determination of Anti-depressant drugs was required for the scheduled pharmacokinetic and pharmacodynamic study of plasma samples. Available methods by means of LC-MS/MS had been published for the simultaneous estimation of these drugs in human plasma in the literature. These reported methods require lengthy steps and extensive labour for sample preparation. As a result undesirable variability of precision and accuracy in quantitative measurements of all analyte at trace levels (ng/ml) can be introduced. I have successfully developed and validated this LC-MS/MS method to meet the sensitivity levels required. As solid phase extraction and liquid-liquid extraction were relatively time consuming and cost affecting when associated with other method called protein precipitation, which was simple, suitable for estimation of analyte within less time and also economical. The volume ratio of 1:3 (analyte sample: methanol containing IS as precipitating agent) was used for sample extraction procedure.

Due to the varying polarities of Duloxetine, developing a chromatographic method encompassing the single analyte proved to be challenging. LC columns including, Phenomenex Synergi™ POLAR RP 80A, were evaluated during the course of chromatographic development and establishment. During method development, it was observed that analyte yielded unacceptable tailing and peak splitting in case of Duloxetine, thus methods utilizing these columns might not be feasible for method validation according to US FDA Guidance for Industry 'Bioanalytical Method Validation'. After having evaluated several columns, X-bridge (4.6×50mm, 3.5µ) was chosen for acceptable chromatography and quantitation. During MS/MS experiments multiple reactions monitoring mode (MRM) was chosen for needed selectivity and sensitivity. Among potential transitions from precursor ion to product/daughter ion, it was noted that compound (Duloxetine hydrochloride), plus one internal standard (Telmisartan) were observed during MS/MS scans. For instance, transitions from 297.90 to 154.10 were evident, which may be used in MRM detections. For internal standards the MRM transitions were from 515.10 to 276.30 for Telmisartan. Selection of the above ion transitions for detection and quantitation was made upon adequate selectivity and sensitivity in the actual K_2EDTA human plasma matrix.

The three method validation batches met all acceptance criteria for linearity (at least 75% of non-zero standards at 85-115% accuracy (80-120% at the LLOQ) and of the regression statistics >0.990) for all analytes. The above method validation batches met all acceptance criteria for precision and accuracy of quality control samples at the LLOQ (at least 2/3 QCs at 80-120%, inter- and intra-batch average accuracy of 80-120%, inter and intra-batch %CV < 20%), inter- and intra-batch at the low mid and high QC levels (at least 2/3 QCs at 85-115% accuracy, inter and intra-batch average accuracy between 85-115%, inter and intra-batch %CV < 15%). The analysis showed no interfering peaks at the retention time of the analytes or internal standards in the specificity from the six individual lots of K_2EDTA human plasma used.

The recovery of the protein precipitation extraction procedure was also evaluated. Extracts of the low, mid and high QC levels were compared with injection of neat standards at the same final concentration. The recoveries were typically within 73-100% for all analytes (97.90% for Duloxetine hydrochloride). Freeze-thaw stability bench-top and auto-sampler stability of low, mid and high quality control samples was validated for freezing and thawing over three cycles and storing samples at auto-sampler respectively. The analytes showed no instability due to freezing and thawing and storage at auto-sampler temperature conditions.

Table No.1: Stock, Spiking Solutions and Matrix CC, QC Preparation Sheet

S.No	Stock, Spiking solutions and Matrix CC and QC Preparation Sheet	
1	Name of the compound	Duloxetine
2	Weight of the compound (mg)	1.916
3	Molecular weight of the free base	297.4
4	Molecular weight of the salt form	333.8
5	% Purity	100
6	Volume of solvents added (mL)	2.16

Preparation of matrix calibration curve standards

S.No	SD B.No Taken	SD Conc. (ng/mL)	SD Vol. (µL)	Diluent Vol. (µL) added	Final. Vol. (µL)	SS Conc. (ng/mL) Prepared	SS Vol. (µL)	Matrix Vol. (µL) added	Final. Vol. (µL)	Final. Conc. In matrix (ng/mL)	STD
Main stock		790308	6.5	994	1000	5137	2	48	50	205.48	STD-8
1	SS-01	5137	900	100	1000	4623.3	2	48	50	184.93	STD-7
2	SS-02	4623.3	750	250	1000	3467.48	2	48	50	138.7	STD-6
3	SS-03	3467.48	550	400	1000	1907.11	2	48	50	76.28	STD-5
4	SS-04	1907.11	320	680	1000	610.28	2	48	50	24.41	STD-4
5	SS-05	610.28	220	780	1000	134.26	2	48	50	5.37	STD-3
6	SS-06	134.26	180	820	1000	24.17	2	48	50	0.97	STD-2
7	SS-07	24.17	500	500	1000	12.08	2	48	50	0.48	STD-1

Preparation of matrix quality control standards

S.No	SD B.No Taken	SD Conc. (ng/mL)	SD vol. (µL)	Diluent vol. (µL) added	Final Vol. (µL)	SS Conc. (ng/mL) Prepared	SS Vol. (µL)	Matrix Vol. (µL) added	Final. Vol. (µL)	Final. Conc. In matrix (ng/mL)	QC
1	Main stock	0	6	994	1000	4741.85	2	48	50	40	HQC
2	HQC	4741.85	300	200	500	2845.11	2	48	50	20	MQC
3	MQC	2845.11	21.1	1478.9	1500	40.02	2	48	50	60	LQC

SD=Stock dilution, SS= Spiking solution, Vol=Volume, Conc=Concentration, STD=Standard, QC=Quality control

Table No.2: Positive ion mode (for Duloxetine hydrochloride)

S.No	Parameter	Value
1	Ionization type and polarity	ESI, positive ion mode
2	Ion source	Turbo spray
3	Scan type	MRM
4	Ion spray voltage	5500V
5	Q1 Resolution	Unit (0.7)
6	Q3 Resolution	Unit (0.7)
7	Temperature	500^0C
8	Gas1	50
9	Gas2	50
10	CUR gas	20
11	CAD gas	4
12	Ihe	ON

Table No.3: Summary of MRM transition conditions

S.No	Compound Name	Mode of ionization	Q1 mass (m/z)	Q3 mass (m/z)	DP	EP	CE	CXP
1	Duloxetine	Positive	298	154	35	10	38	12
2	Telmisartan	Positive	515	276	60	6	64	11

Table No.4: Matrix selectivity for Duloxetine hydrochloride

S.No	Sample Name	Sample Type	Analyte peak area (counts)	Analyte R_t (min.)	IS peak area (counts)	IS R_t (min.)	Area ratio
1	Matrix selectivity-1	Unknown	0	0	4117531	0.87	0
2	Matrix selectivity-2	Unknown	0	0	4226348	0.88	0
3	Matrix selectivity-3	Unknown	0	0	4357381	0.86	0
4	Matrix selectivity-4	Unknown	0	0	4166698	0.87	0
5	Matrix selectivity-5	Unknown	0	0	4088758	0.88	0
6	Matrix selectivity-6	Unknown	0	0	4002402	0.88	0

Table No.5: Back-calculated standard curve data for Duloxetine in human plasma

S.No	Concentration (ng/mL)							
	Std.conc.	Batch-1	Batch-2	Batch-3	Mean	SD	% CV	%Accuracy
1	0.48	0.516	0.463	0.48	0.486	0.027	5.564	101.32
2	0.97	0.812	1.048	0.96	0.94	0.119	12.688	96.91
3	5.37	5.497	5.047	5.5	5.348	0.261	4.874	99.59
4	24.41	26.808	23.791	25.73	25.443	1.529	6.009	104.23
5	76.28	87.323	86.972	79.75	84.682	4.275	5.048	111.01
6	138.7	133.634	128.646	128.76	130.347	2.847	2.185	93.98
7	184.93	159.391	156.642	181.83	165.954	13.817	8.326	89.74
8	205.48	242.606	231.094	200.14	224.613	21.962	9.778	109.31

Table No.6: Calculated concentrations obtained for precision and accuracy batches

S.No	Batch-1			Batch-2			Batch-3		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
1	1.583	108.742	197.419	1.802	126.525	177.031	1.33	95.19	177.61
2	1.786	133.573	195.792	2.021	125.866	189.423	1.4	104.66	170.79
3	1.847	119.43	194.446	1.829	105.655	188.408	1.36	102.43	168.11
4	1.697	119.818	255.989	1.905	101.655	225.226	1.45	99.82	178.9
5	1.844	120.52	234.989	1.648	109.37	210.564	1.48	102.34	173.37
6	1.984	117.01	265.795	1.968	105.281	222.594	1.548	101.42	181.21
Mean	1.79	119.84	224.071	1.862	112.392	202.207	1.428	100.976	174.998

Table No.7: Intra- and inter-run precision and accuracy for Duloxetine in human plasma

S.No	Batches	Intra and Inter-run Precision and Accuracy	Concentration (ng/mL)		
			LQC(1.75)	**MQC**(113.8)	**HQC**(189.67)
1	Batch-1 (N=6)	Intra-run mean	1.79	119.85	224.07
		Intra-run SD	0.14	8	32.45
		Intra-run % CV	7.71	6.68	14.48
		Intra-run % Accuracy	102.3	105.32	118.14
2	Batch-2 (N=6)	Intra-run mean	1.86	112.39	202.21
		Intra-run SD	0.13	10.97	20.02
		Intra-run % CV	7.16	9.76	9.9
		Intra-run % Accuracy	106.4	98.76	106.61
3	Batch-3 (N=6)	Intra-run mean	1.43	100.98	175
		Intra-run SD	0.08	3.24	5.07
		Intra-run % CV	5.65	3.21	2.9
		Intra-run % Accuracy	81.6	88.73	92.26
4	Inter-Batch (N=18)	Inter-run mean	1.69	111.07	200.43
		Inter-run SD	0.23	11	29.36
		Inter-run % CV	13.33	9.91	14.65
		Inter-run % Accuracy	96.77	97.6	105.67

Table No.8: Duloxetine recovery data

S.No	LQC Area (Counts)		MQC Area (Counts)		HQC Area (Counts)	
	Aqueous	**Extracted**	**Aqueous**	**Extracted**	**Aqueous**	**Extracted**
1	5293	5041	313662	326222	690696	582361
2	5035	5120	320805	340742	607426	571255
3	5078	4783	312970	349177	583222	566924
4	5431	5284	317530	335456	627983	585855
5	5336	5150	313664	334632	629201	561793
6	5337	5121	335525	340741	607427	561792
Mean Area	52346	50756	320098	337246	627706	573638
Recovery	96.9625186		105.356915		91.3864079	
% Recovery (Average) = 96.9625186						
SD = 7.032						
% CV = 7.18						

Table No.9: Showing recovery data of Duloxetine

S.No	Analyte	Concentration			Avg. Recovery	SD	% CV
		LQC	**MQC**	**HQC**			
1	Duloxetine	96.9	105	91.4	97.9	11	10.76

Table No.10: Stability data showing the % CV of analytes

S.No	Analyte Name	Fresh Samples	Freeze- thaw stability samples	Auto-sampler stability samples	Bench - top stability samples
1	Duloxetine	-	-	-	-
2	LQC	7.874	7.886	13.46	7.879
3	MQC	6.68	6.681	6.68	6.681
4	HQC	7.026	7.028	16.102	12.33

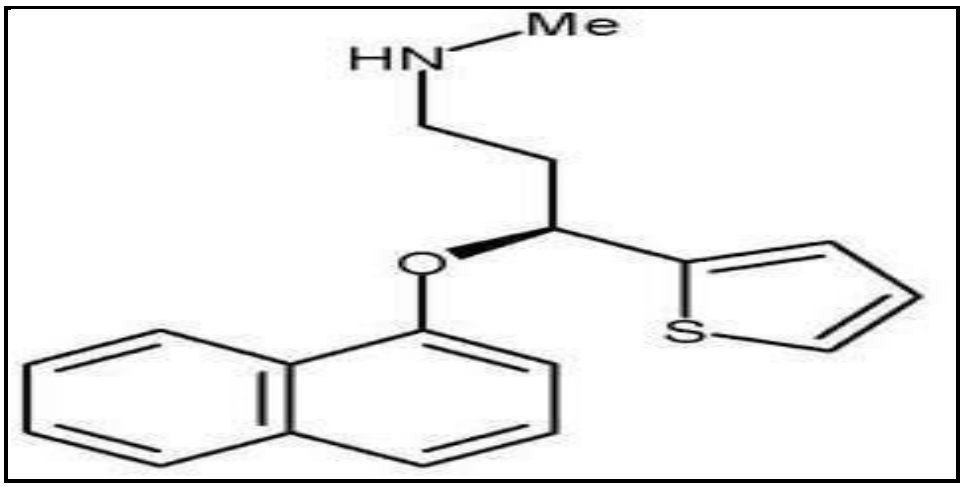

Figure No.1: Structure of Duloxetine Hcl

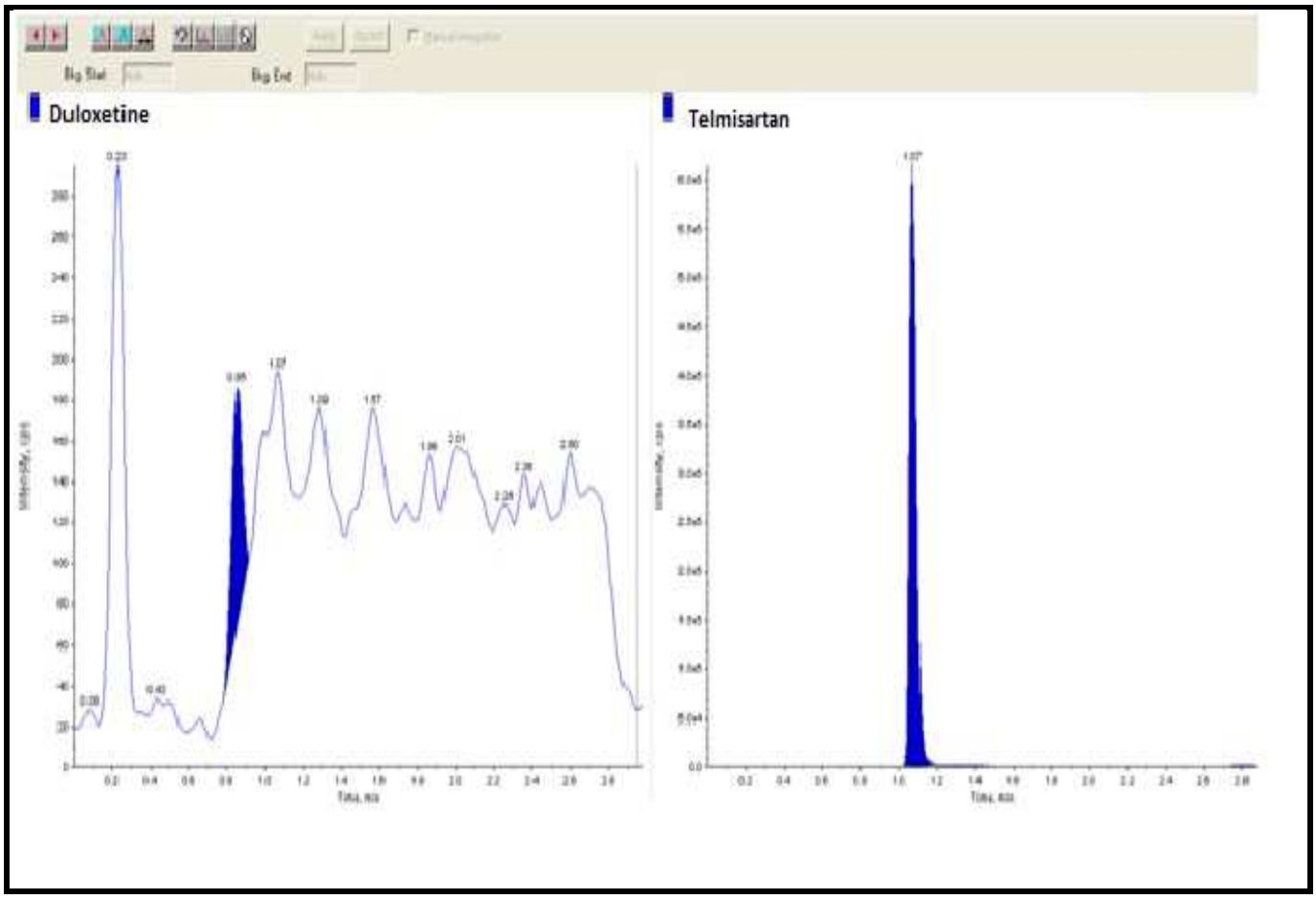

Figure No.2: Optimization of Chromatogram for Duloxetine Hydrochloride for Trail-1

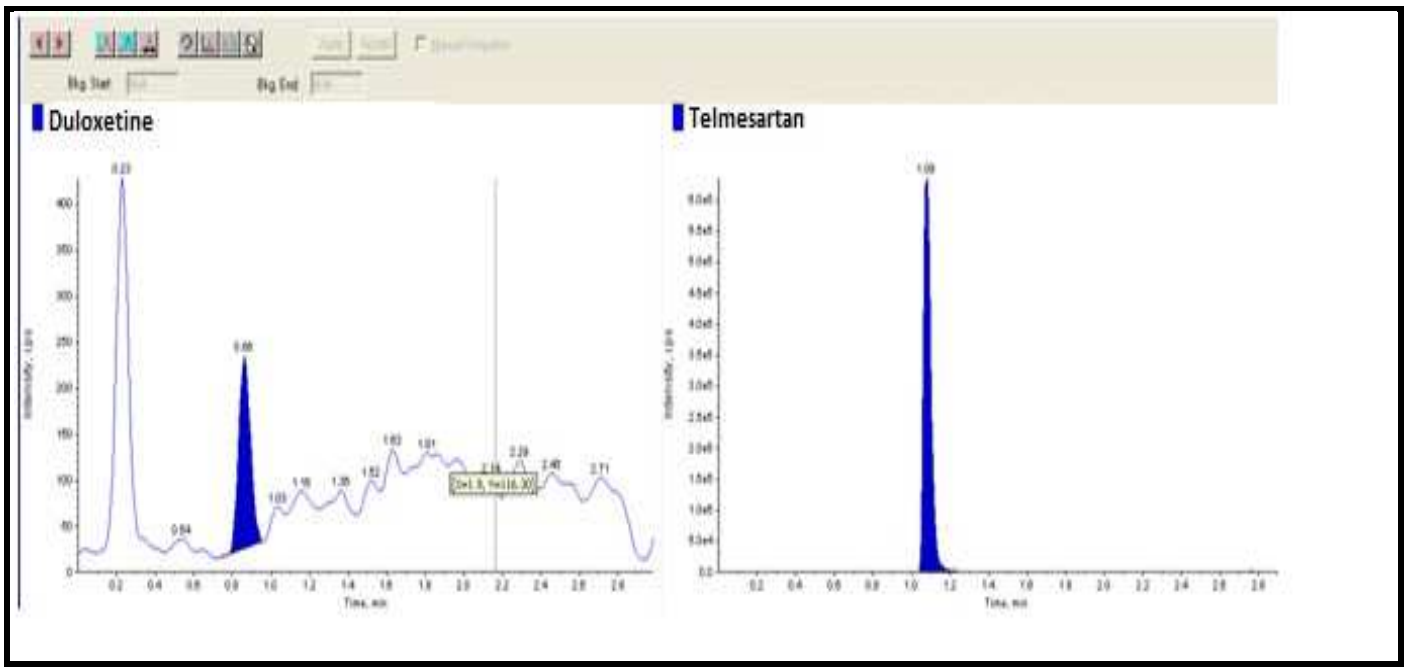

Figure No.3: Optimization of Chromatogram for Duloxetine Hydrochloride for Trial-2

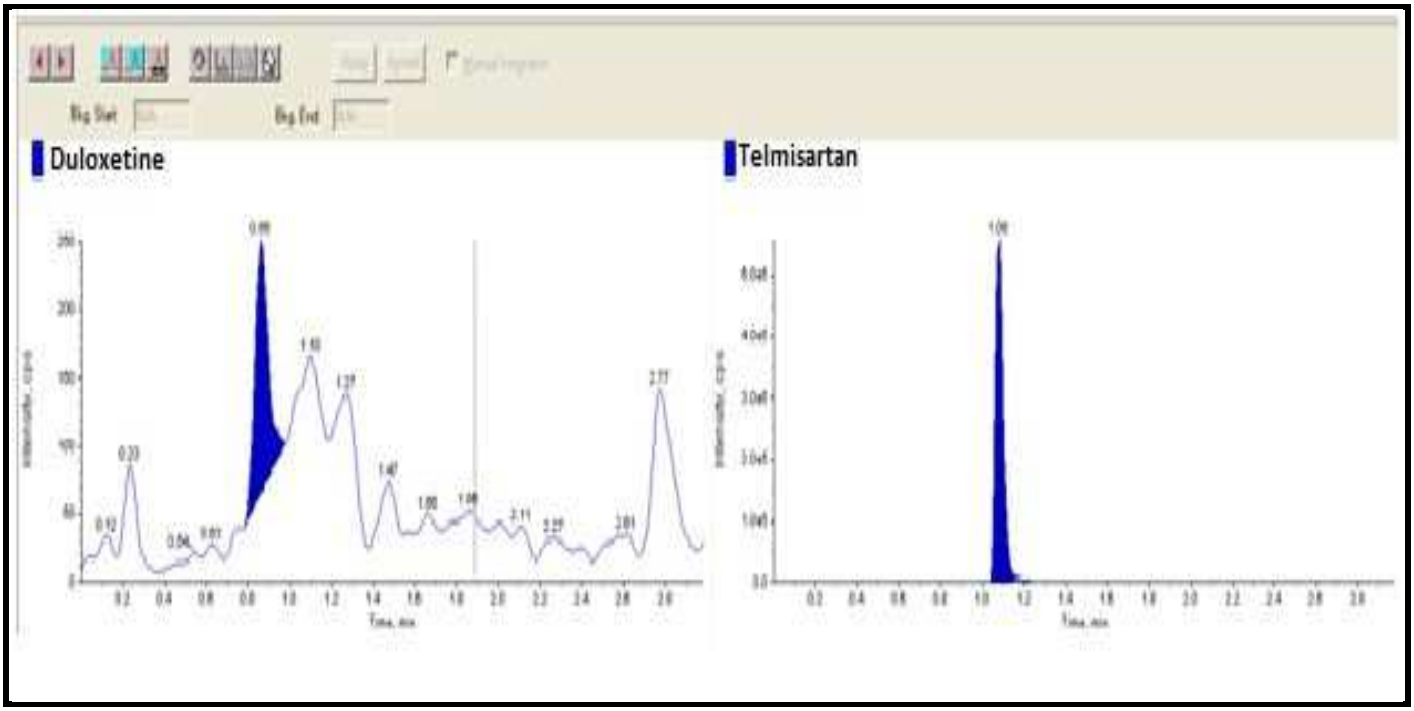

Figure No.4: Optimization of Chromatogram for Duloxetine Hydrochloride for Trial-3

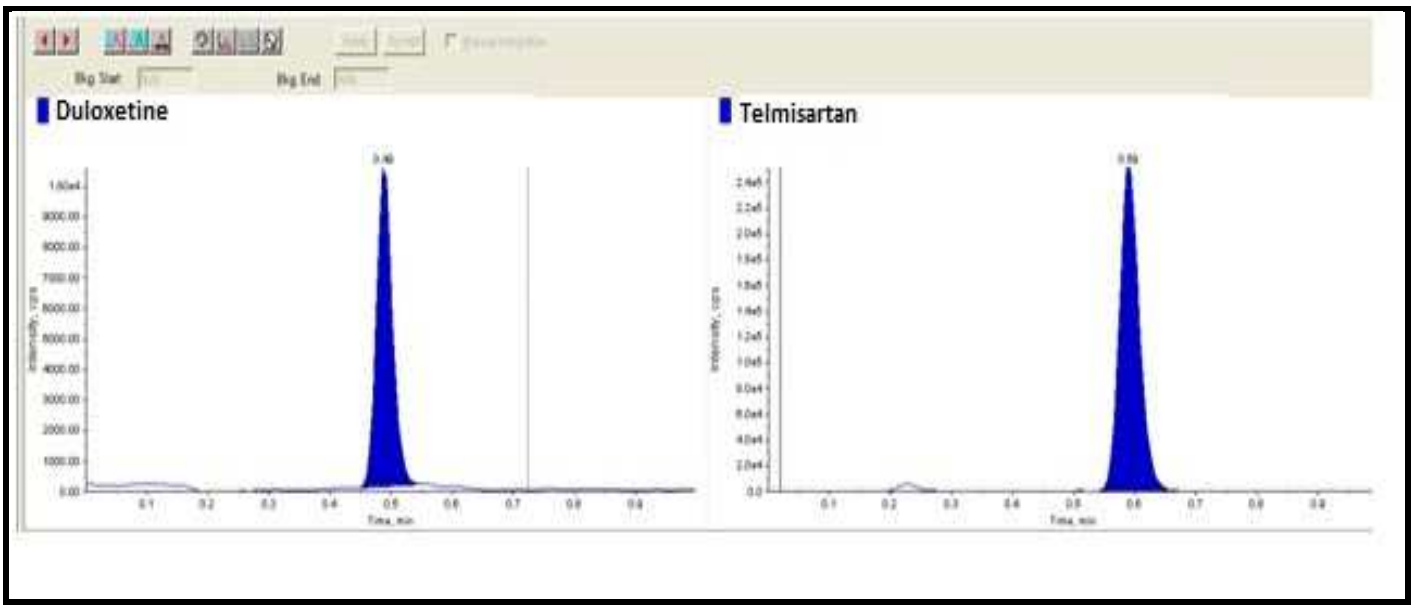

Figure No.5: Final Optimization of Chromatogram for Duloxetine Hydrochloride

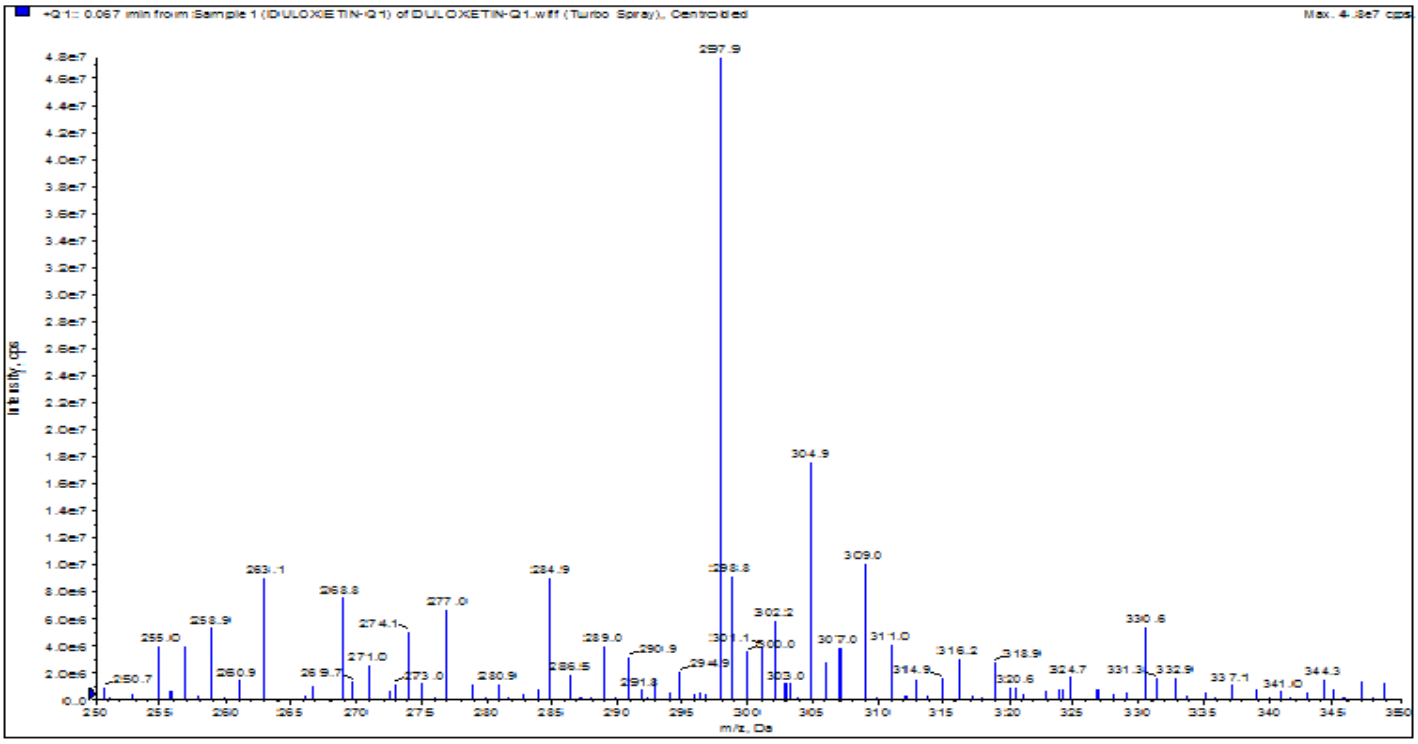

Figure No.6: Duloxetine Q3 Ms Scan Tune Window Showing Abundant Parent Ion At M/Z of 154.10 Comparison of Duloxetine Product Ions And Their Intensities (Q1/Q3) Showing M/Z of 297.90/154.10

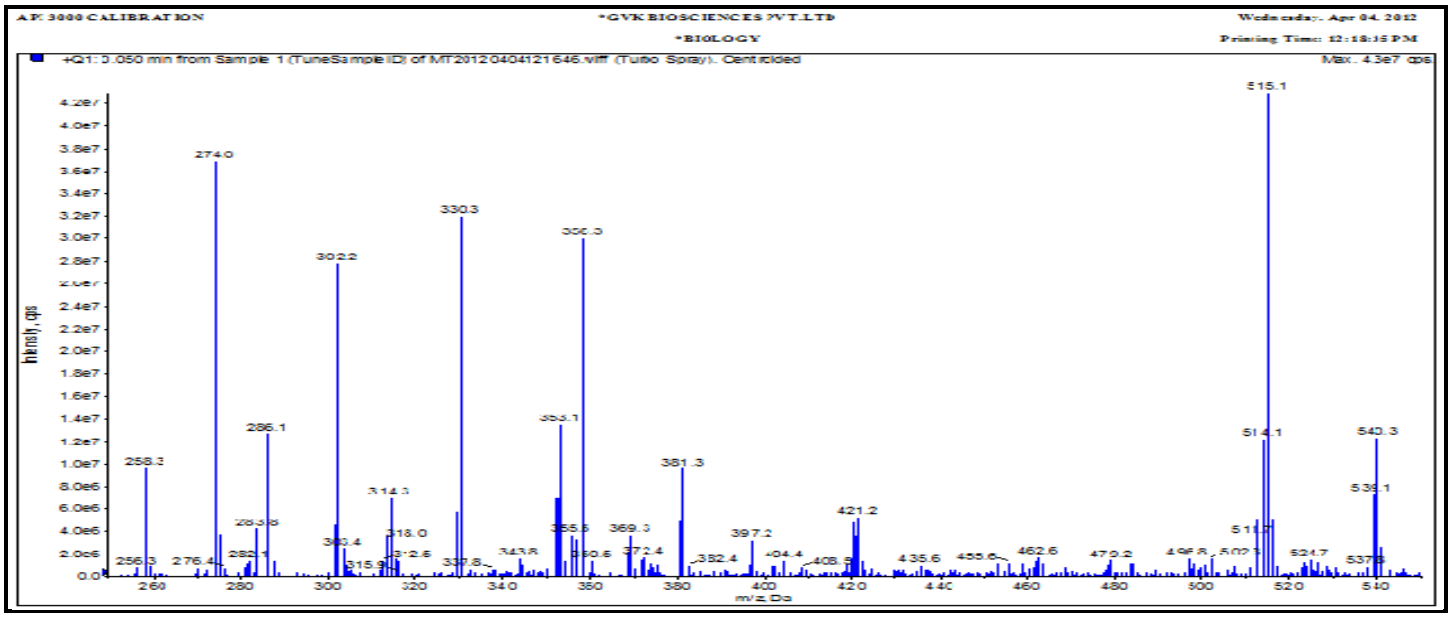

Figure No.7: Telmisartan Q1 MS scan tune window showing abundant Parent ion at m/z of 515.1

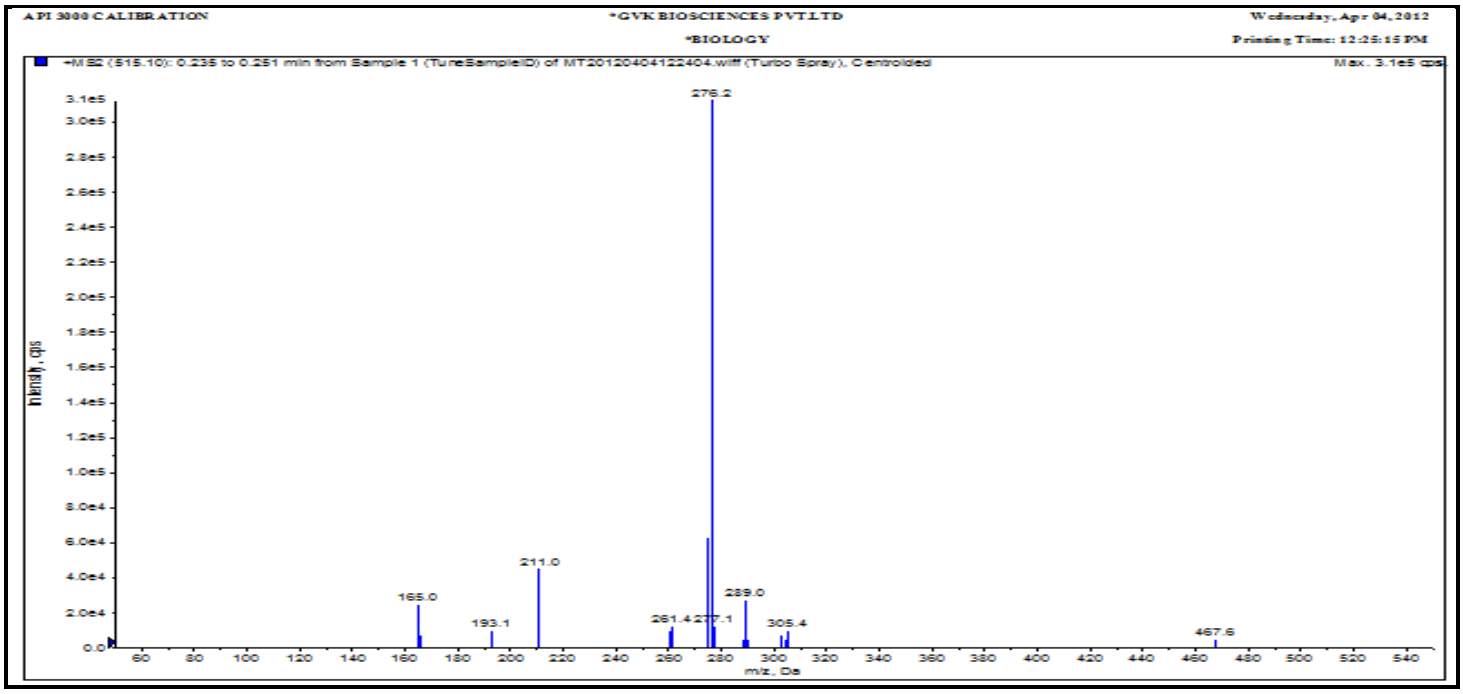

Figure No.8: Telmisartan Q3 MS scan tune window showing abundant Parent ion at m/z of 276.2
Comparison of Telmisartan product ions and their intensities (Q1/Q3) showing m/z of 515.1/276.2

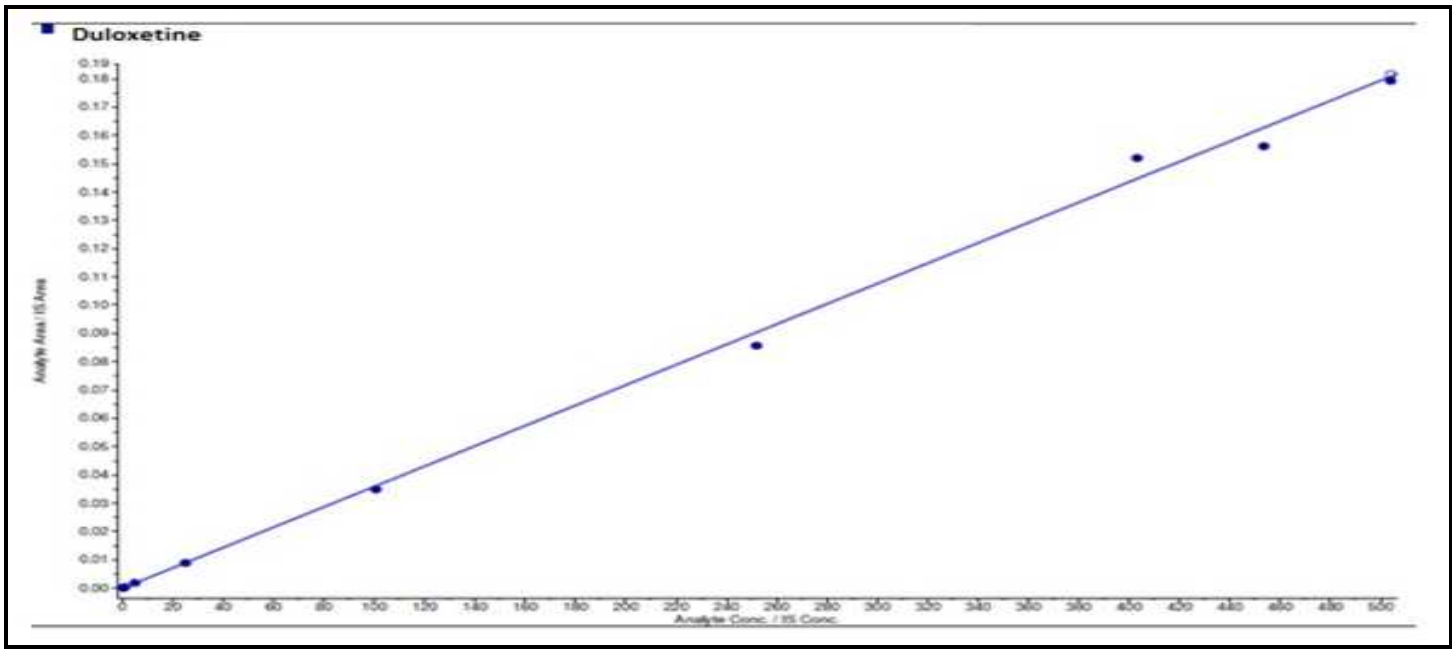

Figure No.9: Representative example of calibration curve for Duloxetine

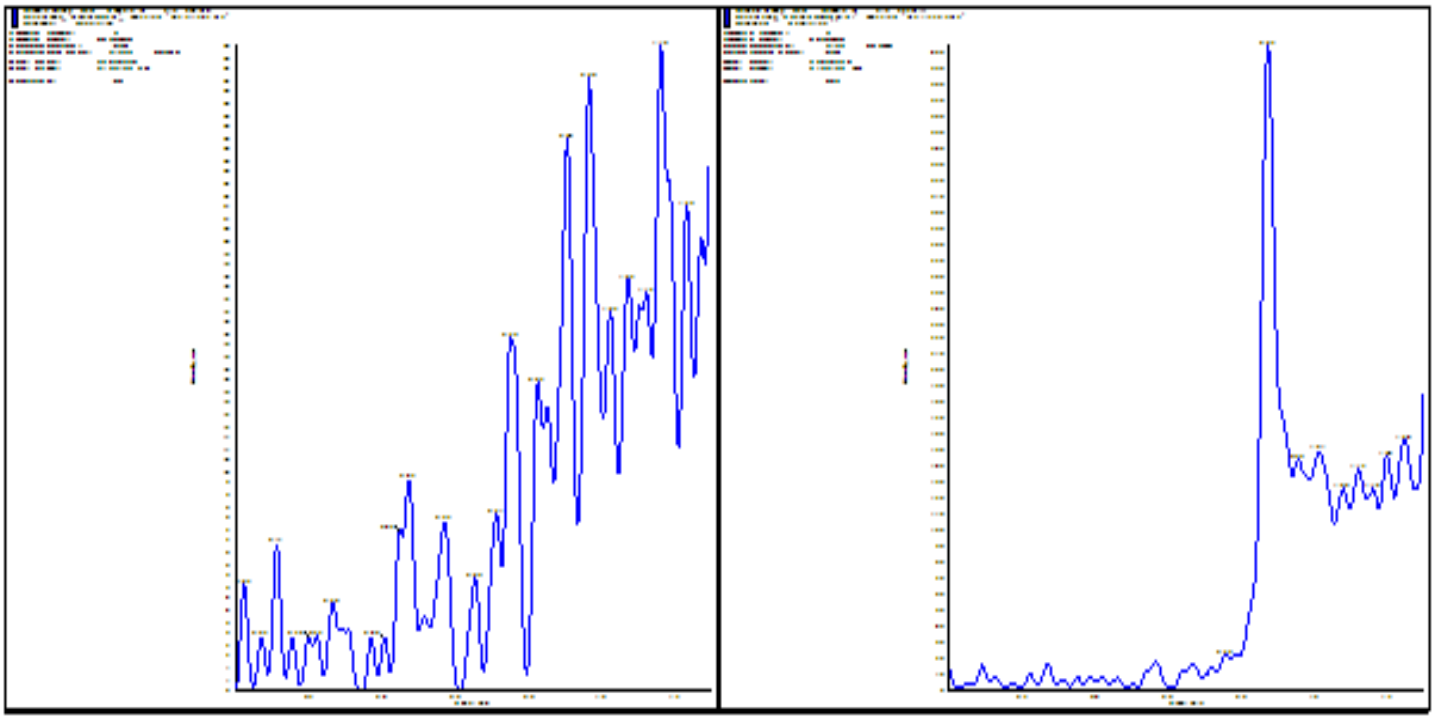

Figure No.10: Representative example of blank chromatogram for Duloxetine

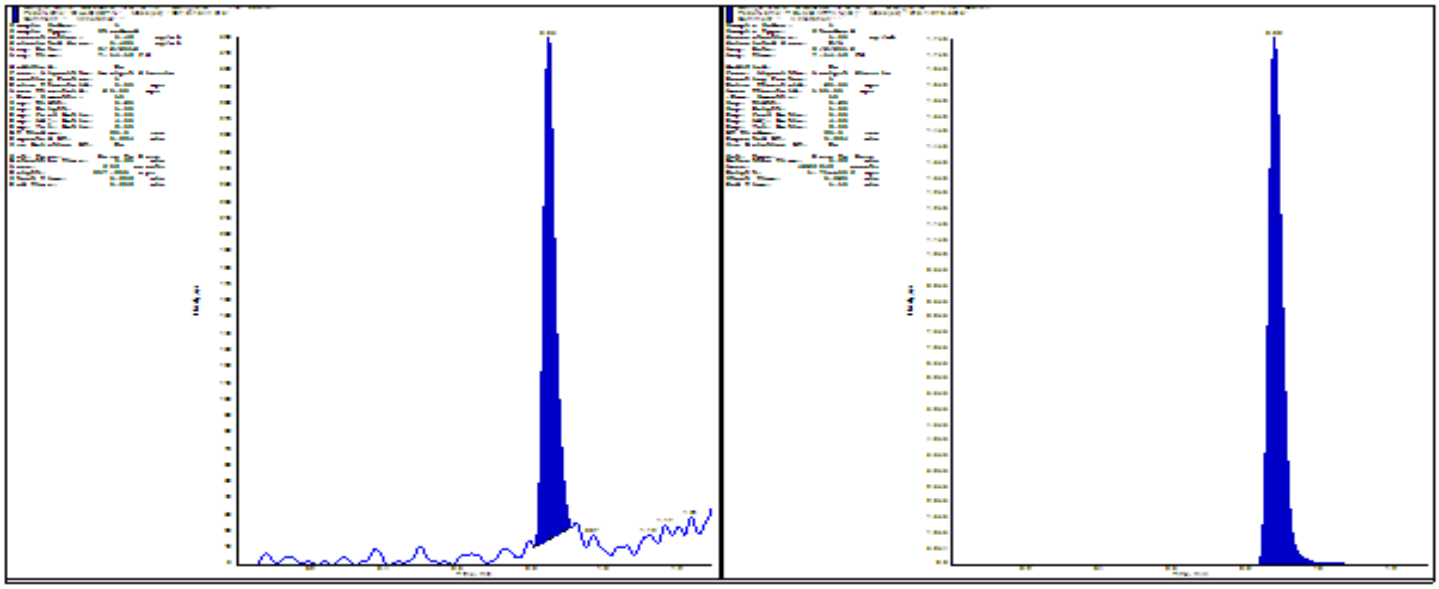

Figure No.11: Representative example of STD-1 chromatogram for Duloxetine

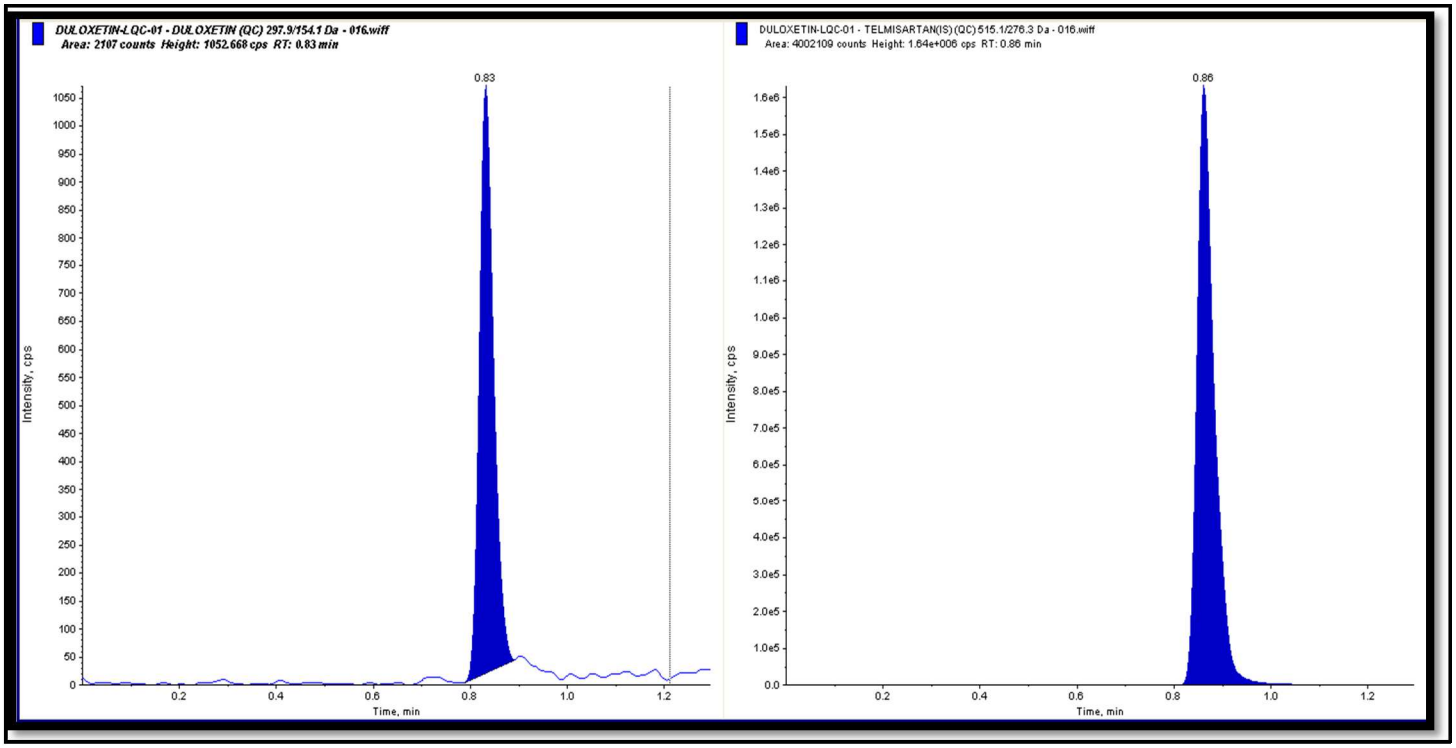

Figure No.12: Representative example of LQC chromatogram for Duloxetine

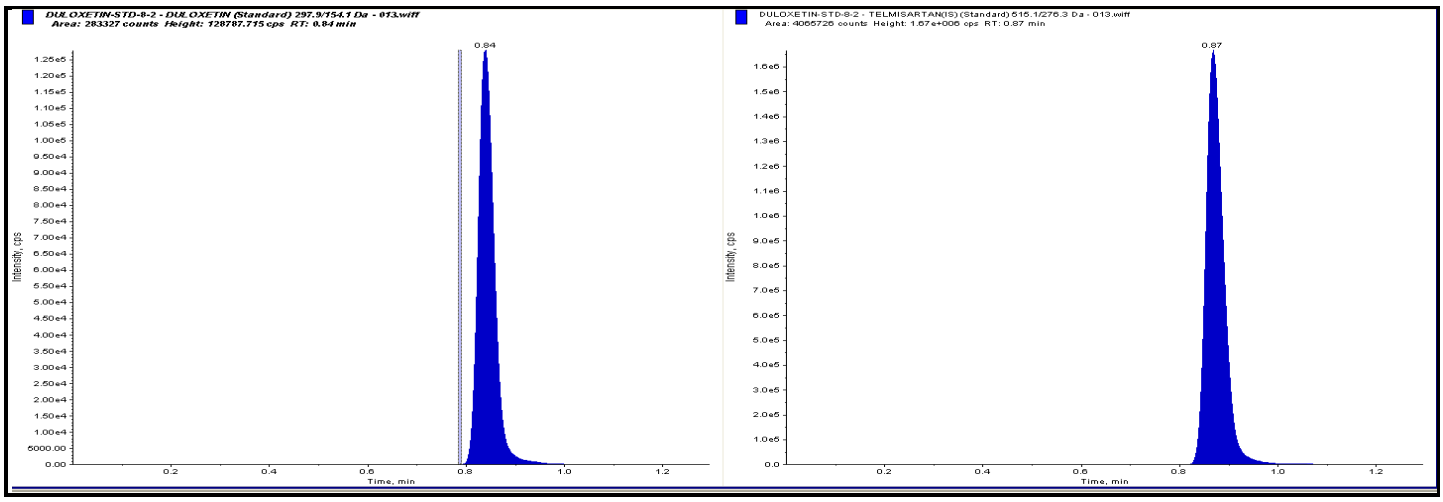

Figure No.13: Representative example of MQC chromatogram for Duloxetine

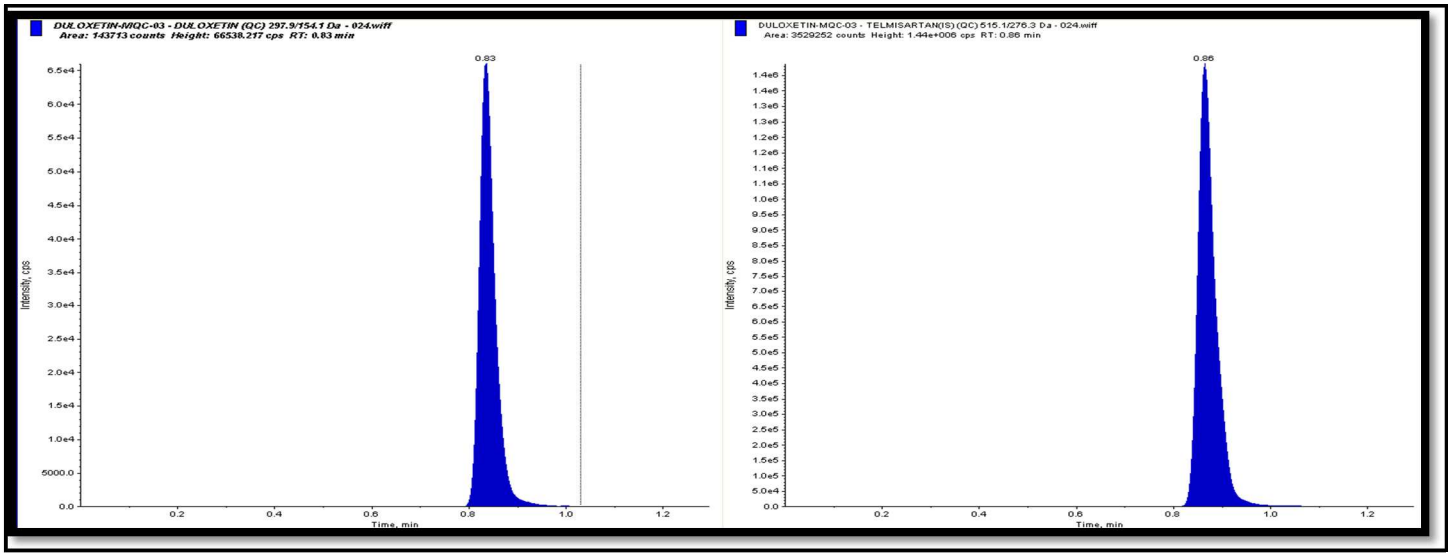

Figure No.14: Representative example of HQC chromatogram for Duloxetine

CONCLUSION

A simple, sensitive, specific, economical and fast LC-MS/MS method has been developed and validated for estimation of Duloxetine hydrochloride in human plasma. The use of a protein precipitation clean-up stage gives simplicity of application and is capable of processing no. of samples with in short time. Intra-assay and inter-assay variability has also shown the capability of the method described here to provide reproducible determination of analytes in a matrix. Moreover, significant decrease in analysis time would lead to increased throughput of samples providing a more viable approach for estimation of analyte than is currently available. The method showed good applicability for measuring these drugs in plasma samples.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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