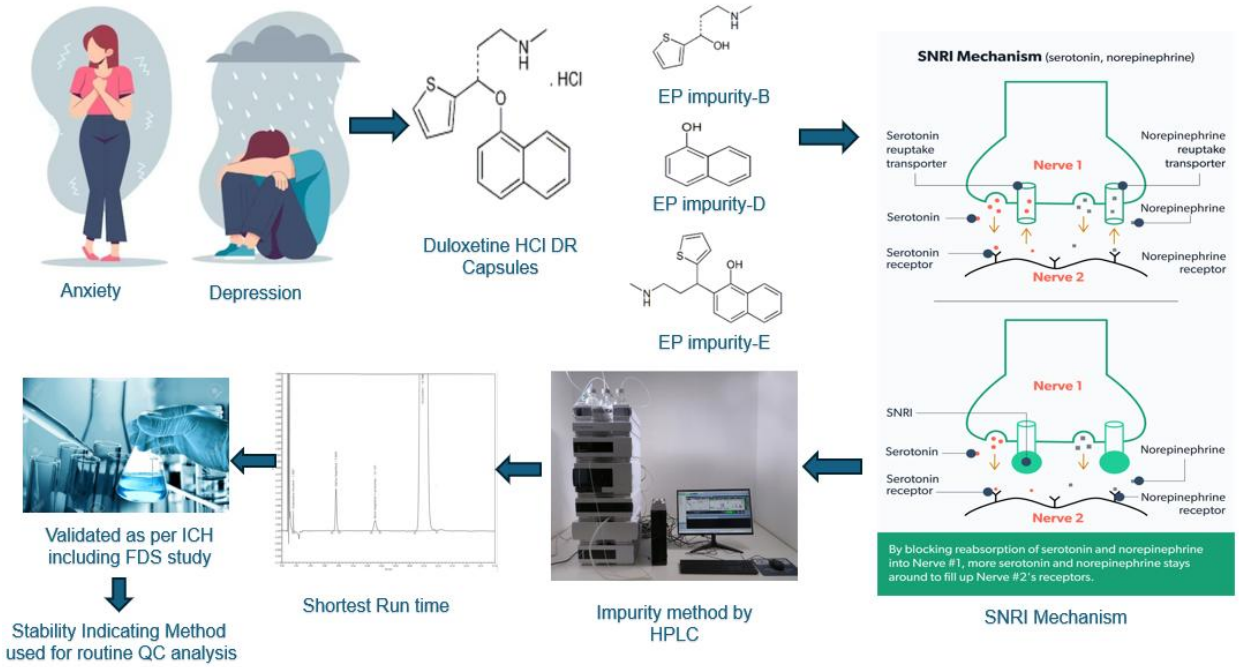
International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 15s, 2025
https://www.theaspd.com/ijes.php

Development and validation of reversed phase chromatography method for the determination of process and degradation impurities in Duloxetine Hydrochloride delayed release capsules

***Ramesh Nethala*[1*], *Anjanapura V Raghu*[2], *K.Anand Solomon*[3], *Mallikharjuna Rao Bandlamudi*[4], *Srinivas Murthy V*[5], *Chaitanya Balusu*[6]**

[1*]*Department of Chemistry, School of Engineering, Dayananda Sagar University, Devarakaggalahalli, Harohalli, Kanakapura Road, Ramanagara District - 562 112.*
[2]*Department of Chemistry, School of Engineering, Dayananda Sagar University, Devarakaggalahalli, Harohalli, Kanakapura Road, Ramanagara District - 562 112*
[3]*Department of Chemistry, School of Engineering, Dayananda Sagar University, Devarakaggalahalli, Harohalli, Kanakapura Road, Ramanagara District - 562 112.*
[4]*Chief Executive Officer, Qdot Associates (Pharma Consulting Company), Hyderabad,500090, India.*
[5]*Department of Chemistry, School of Engineering, Dayananda Sagar University, Devarakaggalahalli, Harohalli, Kanakapura Road, Ramanagara District - 562 112.*
[6]*Department of Chemistry, School of Engineering, Dayananda Sagar University, Devarakaggalahalli, Harohalli, Kanakapura Road, Ramanagara District - 562 112.*

***Corresponding Author:** Ramesh Nethala*
***Email**: rameshnethala@gmail.com*



Submission date: 29-05-2025; Acceptance date: 10-07-2025

Abstract
Duloxetine Hydrochloride is a delayed-release serotonin and norepinephrine reuptake inhibitor (SNRI) administered orally, utilized for the treatment of depression and anxiety by enhancing the levels of mood-regulating neurotransmitters, serotonin and norepinephrine, in the brain. Drug classified as BCS Class-II, having low solubility and high permeability. The dissociation constant (pKa) of Duloxetine Hydrochloride was determined to be 9.7, and the molecule is strong basic in nature. However,

International Journal of Environmental Sciences
ISSN: 2229-7359
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Gradient methods with longer runtimes were published to determine the selective impurities of Duloxetine. The study reported here is an isocratic method with the shortest possible run time with selective impurities (process and degradation impurities) of Duloxetine. Forced degradation studies were performed on Duloxetine Hydrochloride Capsules using Acid, Base, Oxidative (Peroxide), Humidity, Thermal and Photolytic stress conditions. The identified unknown degradants were well resolved from the known impurities and the mass balance found close to 100% in all the stress conditions applied. The chromatographic technique was optimized utilizing stress samples from stress degradation investigations. The analytical method was validated in accordance with ICH requirements. The obtained validation results demonstrate the developed analytical method can be employed in routine chemical analysis for the determination of known impurities i.e. Duloxetine alcohol impurity (Duloxetine EP impurity B), Duloxetine Alpha Naphthol impurity (Duloxetine EP impurity D) and Duloxetine β-Naphthol-1-yl-isomer (Duloxetine EP impurity E) and unknown impurities (any unspecified impurities).

Keywords: *Duloxetine HCl, Forced Degradation Study (FDS), Confidence interval, Relative Standard Deviation (RSD), LOD (Limit of Detection), LOQ (Limit of Quantification) and BCS (Biopharmaceutics Classification System)*

1.Introduction

Depression is a frequent yet severe mental disease. It causes severe symptoms that affect how people feel, think, and sleep, eat, and work [1]. Depression differs from typical mood fluctuations and sentiments of daily existence. It can influence all facets of life, including connections with family, friends, and the community. In 2021, about 61.0% (or 12.6 million individuals) of US adults aged 18 and older diagnosed with serious depressive disorders got treatment. Approximately 74.8% of individuals experiencing major depressive episodes with severe impairment received treatment in the previous year [2]. About 280 million people worldwide suffer from depression[3]. The WHO reports that around 3.8% of the population experiences depression, which includes 5% of adults (4% of men and 6% of women) and 5.7% of individuals over 60 years old [4]. Despite the presence of effective therapies for mental disorders, nearly 75% of individuals in low and middle-income countries do not receive any treatment [3-6]. Duloxetine Hydrochloride is a significant antidepressant, with a market valuation of USD 2.32 billion in 2023, projected to increase to USD 3.76 billion by 2030. This represents a compound annual growth rate (CAGR) of 6.07% from 2024 to 2030 [7].

Duloxetine is used to treat certain mental/mood disorders (such as depression, anxiety). It is also used to treat pain produced by nerve damage linked with diabetes (diabetic peripheral neuropathy) in persons with diabetes, as well as pain caused by medical diseases such as arthritis, chronic back pain, or fibromyalgia [8]. Duloxetine, which binds to human serum albumin, treats Major Depressive Disorders (MDD) safely and effectively[9-11]. Duloxetine Hydrochloride chemical name is (γS)-N-Methyl-γ-(1-naphthalenyloxy)-2-thiophenepropanamine Hydrochloride with empirical formula $C_{18}H_{20}ClNOS.HCl$ (*Figure-1*). The Literature Survey on Duloxetine Hydrochloride reveals that many articles were published on quantification of Duloxetine hydrochloride in active pharmaceutical ingredients (API), bulk and finished dosage form [12-20]. Nevertheless, relatively few articles have been published using gradient chromatographic elution with extended run times on selected impurities [21-25]. However, no isocratic approach with the shortest run time for separating process and degrading impurities of Duloxetine has been disclosed. In the current study attempts were made to develop a sensitive, accurate, precise, robust and stability indicating method (SIM) with selective impurities and degradation products in shortest run time.

The impurities selected for the study are Duloxetine alcohol impurity (Duloxetine EP impurity B), Duloxetine Alpha Naphthol impurity (Duloxetine EP impurity D), and Duloxetine β-Naphthol-1-yl-isomer (Duloxetine EP impurity E) (*Figure-1*). Duloxetine alcohol impurity (Duloxetine EP impurity B) bearing a chemical name (1S)-3-(Methylamino)-1-(thiophen-2-yl) propan-1-ol with empirical formula $C_8H_{13}NOS$.Duloxetine alcohol impurity is a process related impurity which is considered as category specified impurity. Duloxetine Alpha Naphthol impurity (Duloxetine EP impurity D) bearing a chemical name Naphthalen-1-ol with empirical formula $C_{10}H_8O$. Duloxetine Alpha Naphthol impurity is a Degradant impurity which is considered as specified degradation impurity. Duloxetine β-Naphthol-1-yl-isomer (Duloxetine EP impurity E) bearing a chemical name of 2-[(1RS)-3-(Methylamino)-1-(thiophen-2-yl) propyl] naphthalen-1-ol with empirical formula $C_{18}H_{19}NOS$. Duloxetine β-Naphthol-1-yl-isomer is a process related which is considered category specified impurity. The Total Daily Intake limit of these impurities is 0.2%, Individually [26-27].

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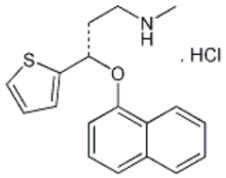
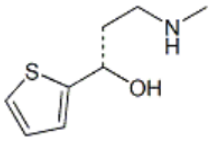
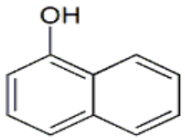
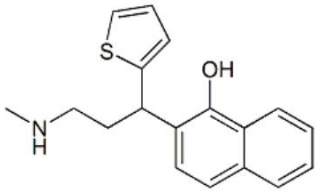
Figure 1: Chemical Structure of Duloxetine Hydrochloride and its Impurities

Duloxetine Hydrochloride	Duloxetine alcohol impurity (Duloxetine EP impurity B)	Duloxetine Alpha Naphthol impurity (Duloxetine EP impurity D)	Duloxetine β-Naphthol-1-yl-isomer (Duloxetine EP impurity E)
			
Mol.Wt: 333.88 g/mol (CAS No.: 136434-34-9)	Mol.Wt: 171.26 g/mol (CAS No.: 116539-55-0)	Mol.Wt: 144.17 g/mol (CAS No.: 90-15-3)	Mol.Wt: 297.41 g/mol (CAS No.: 1033803-59-6)

Developed an Isocratic reversed phase chromatographic method with a run time of 30 min and performed analytical method validation as per ICH recommendations [28-29]. The mass balance was determined to be almost 100% under all applied stress situations. None of the selected impurities (Impurity B, D, E) and potential degradation products generated through the stress degradation conditions were co-eluting with each other and confirmed that the analyte peak was homogeneous and pure. The developed method is confirmed as a stability-indicating method (SIM) and is suitable for use in the stability monitoring of the drug product Duloxetine hydrochloride delayed release capsules [30-32]. Therefore, the established method can be used for quantifying impurities (both process and degradation) as well as the content of Duloxetine (assay) in Duloxetine hydrochloride delayed release capsules.

2. Experimental

2.1. Materials

2.1.1 Standards, Impurities, Chemicals and Solvents

Impurities with potency 99.70% for Duloxetine Alcohol impurity, 95.87% for Duloxetine Alpha Naphthol impurity and 99.57% for Duloxetine β-Naphthol-1-yl-isomer was used for the study. The selected three impurities are procured from Laurel Pharma labs with characterization parameters proton NMR, FT-IR and molecular weight by Mass (MS).The Duloxetine Hydrochloride working standard with potency 99.9% which is qualified against the USP reference standard lot, having batch number R043X0 and Duloxetine Hydrochloride delayed released capsules of 60 mg strength was selected for the study. The chemicals and solvents used for the study were detailed in Table 1.

Table 1: Details of Chemical and solvents used for the study

Name	Grade
1-Heptane Sulphonic acid sodium salt	HPLC
Ortho phosphoric acid	A. R
Sodium hydroxide	HPLC
Methanol	HPLC
Iso-propyl alcohol	HPLC
Water	HPLC

2.2. Instrumentation and method

The study on development and validation was conducted utilizing the Agilent HPLC system [1200 Infinity II LC System] equipped with a Diode Array Detector (DAD). The output signal was monitored and processed using Empower 3 software, version 3.6.1. The chromatographic parameters are detailed in Table 2.

Table 2: Chromatographic Parameters

Chromatographic Particulars	Set Conditions
Column	Hypersil BDS C8,150x4.6mm,5µm.
Flow rate	2.0 mL/min
Injection volume	20 µL
Column temperature	40°C
Sample Compartment temperature	25°C
Wavelength	230 nm
Run time	30 mins

2.3. Preparation of Analytical Solutions

2.3.1 Preparation of Mobile phase and diluent

A buffer solution was prepared by adding 1.7 mL of ortho-phosphoric acid to 1,000 mL water in a container and mixed well. The solution was adjusted to a pH of 2.5 using sodium hydroxide solution, followed by the addition of 10.3 grams of 1-Heptane Sulphonic acid sodium salt (ion pair reagent), and then thoroughly mixed. For the isocratic preparation of the mobile phase, blend buffer, methanol, and isopropyl alcohol in a container in the volume ratio of 700:130:170, respectively, and mix thoroughly. The diluent for the analytical solution preparations was prepared through the addition of water and methanol in a 50:50 v/v ratio.

2.3.2 Standard solution

A Duloxetine Hydrochloride working standard solution of 2 ppm was prepared in a diluent corresponding to the concentration of the test sample.

2.3.3 Sample Solution

Mixed the contents of 20 capsules of Duloxetine Hydrochloride delayed released capsules of 60 mg and from which weighed 25 mg equivalent sample into a 50 mL volumetric flask, added 20 mL of diluent and sonicated for 30 minutes with intermediate shaking and kept aside for attaining room temperature. After that, add diluent and mix thoroughly. After centrifuging a sample at 3000 rpm for 10 minutes, inject the supernatant into the chromatographic system. The end sample solution concentration is 500 ppm in diluent.

2.3.4 Placebo Solution

Weighed 25 mg equivalent of placebo into a 50 mL volumetric flask, added 20 mL of diluent and sonicated for 30 minutes with intermediate shaking and kept aside for attaining room temperature. After that, add diluent and mix thoroughly. After centrifuging a sample at 3000 rpm for 10 minutes, inject the supernatant into the chromatographic system. The end sample solution concentration is 500 ppm in diluent.

2.4 Results and discussion

2.4.1 Analytical Method Validation procedure

Specificity or Selectivity of a method is demonstrated by injecting and identifying the retention times (RT) of standard and impurity peaks along with blank and placebo peaks (if any), that are not interfering with each other from each individual solution.

Precision parameters were demonstrated with respect to System precision, Method precision and Intermediate precision. System precision investigated by using standard solution and confirms through the system suitability criteria are met with predefined acceptance criteria as per USP<621>. The method precision parameter was established by preparing six homogeneous samples individually, following the test method. This was achieved by spiking each sample with all impurities, either individually or as a blend of impurity stock solutions, at their specified levels.

Injected the solutions into the chromatographic system as per the test method, calculated the % of individual impurity, %RSD and confidence interval at 95% level. A similar approach was taken for Intermediate precision

with different analysts, different HPLC system, different lot of columns, different day and with fresh six individual preparations.

Determined LOD and LOQ values for Duloxetine hydrochloride (analyte peak) and its impurities by spiking at lowest concentration level in placebo solution to obtain a signal to noise ratio of 3:1 and 10:1 for LOD and LOQ respectively. Performed LOQ precision by preparing six test solutions by spiking impurities at LOQ level on placebo solution prepared as per test method and calculated the %RSD from six spiked solutions. Performed recovery assessments at the limit of quantitation (LOQ) level in triplicate by spiking impurities at the LOQ level into a placebo solution prepared according to the test procedure and computed the percentage recovery from the spiked solutions.

The linearity of detector response was demonstrated by preparing spiked impurity blend solutions in diluent at different concentration levels from LOQ to 150 % of impurity specification limits. All these solutions were prepared by diluting impurity stock solutions to prepare an impurity mixture and spiked this impurity mixture at different concentration levels from LOQ to 150 % of targeted test concentration in placebo solutions. A linear regression equation was established utilizing the impurity peak area (y-axis) in relation to the estimated concentration in ppm (x-axis). Report the slope, intercept value, and residual sum of squares (R^2) from the regression line.

Accuracy study was performed in the concentration range from 50 % to 150 % of the targeted test concentration. Prepared test sample solutions in triplicate at each level and spiked with impurity stock solutions from 50 % level to 150 % level of targeted test concentration i.e., from 0.5 ppm to 3 ppm level. Calculated the % Recovery of each impurity at each spiked level.

Established the stability of test solutions in 48 hours (2 days) at room temperature. For the study, injected blank, standard and test sample solutions spiked with Impurity blend solution at specification level (0.2%) and injected into chromatographic system for Initial day, day-1, and day-2 analysis. Calculated the % of individual impurities from the spiked test preparation at initial, day-1 and day-2 against freshly prepared standard solution. The % difference in result between the initial to day-1 and day-2 shall be not more than 0.05 absolute value considered as stable.

To establish the test method should be specific for the analyte or peak of interest (impurity), i.e., non-interference of impurities, degradation products and excipients, conducted forced degradation study (FDS) to obtain degradation products, wherever degradation possible from about 5 % to 20 % in at least one stress condition. For conducting forced degradation studies, we used both placebo and test sample for stress study. For acid hydrolysis, used 0.1N Hydrochloric acid (HCl), added to the test solution and placebo solution, kept at room temperature for 6 days and proceeded as per sample preparation procedure. For base hydrolysis 0.1N Sodium Hydroxide (NaOH), added to the test solution and placebo solution, kept on water bath at 60°C for 24 h and proceeded as per sample preparation procedure. For Oxidative stress study, used 3% Hydrogen peroxide (H_2O_2), added to the test solution and placebo solution, kept at room temperature for 3 h and proceeded as per sample preparation procedure. For Humidity stress study, the finished product sample and placebo exposed to 75% Relative Humidity (RH) about 24 h and then proceeded as per sample preparation procedure. For the thermal stress study, we kept the finished product sample and placebo at 60°C for 24 h and then prepared the sample solution as per sample preparation procedure. For Photolytic stress study both finished product sample and placebo, exposed to 200-Watt hours/m^2 for UV light and /1.2 million lux hours for Visible light and then proceeded as per sample preparation procedure. Along with the above stress samples injected control sample (unstressed test sample) in sequence for calculation of Mass balance. To prove it as Stability Indicating Method (SIM), calculated the % assay result from stressed samples and control sample (unstressed sample) and % impurities from stressed samples for computing mass balance. All stressed samples had a mass balance close to 100%, which is consistent with the approved method recognized as the Stability Indicating Method (SIM).

2.4.2. Method Development and Optimization

Compendial method is already available in USP monograph for determination of Duloxetine related impurities by HPLC (Duloxetine EP Impurity H, Duloxetine EP Impurity D and Duloxetine EP Impurity F). In current study, we have considered other than USP monograph method impurities i.e., Duloxetine EP Impurity B, Duloxetine EP Impurity D and Duloxetine EP Impurity E. Alternative analytical processes may be employed for control purposes, providing they provide a definitive determination of compliance with the monograph standards, assuming the official procedures were applied to the intended formulation. To implement alternate procedures other than compendial procedures at site, method validation as per ICH requirements and method equivalency needs to be established to justify the developed method is equivalent and/or superior to the compendial procedure.

In the development of a method for Duloxetine hydrochloride delayed release capsules content and related substances, various factors were evaluated. These include the physical and chemical properties of Duloxetine Hydrochloride, such as pKa values, molecular size and weight, sample solubility, sample volatility, stability and toxicity, hydrophobicity/polarity, chemical reactivity, and the UV spectra of the compound. Mode of separation technique by Chromatography using different mobiles phases, different columns and different chromatographic conditions were considered. The method was optimized to provide a decent separation between the contaminants and the analyte peak in the shortest possible run time.

Initially buffer solutions prepared with mixed phosphate buffers at pH 4.8. The mobile phase is prepared by combining buffer and methanol at a 70:30 v/v ratio. The diluent is made by combining water and methanol in a 50:50 v/v ratio. On a chromatography column Agilent C18, 250 x 4.6mm, 5µm at flow rate 1.6 mL/min with injection volume 10 µL at detection wavelength of 230 nm shows 'Beta Napthol-1-yl-isomer impurity (Duloxetine EP impurity E) merges with the Duloxetine analyte peak'.

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ISSN: 2229-7359
Vol. 11 No. 15s, 2025
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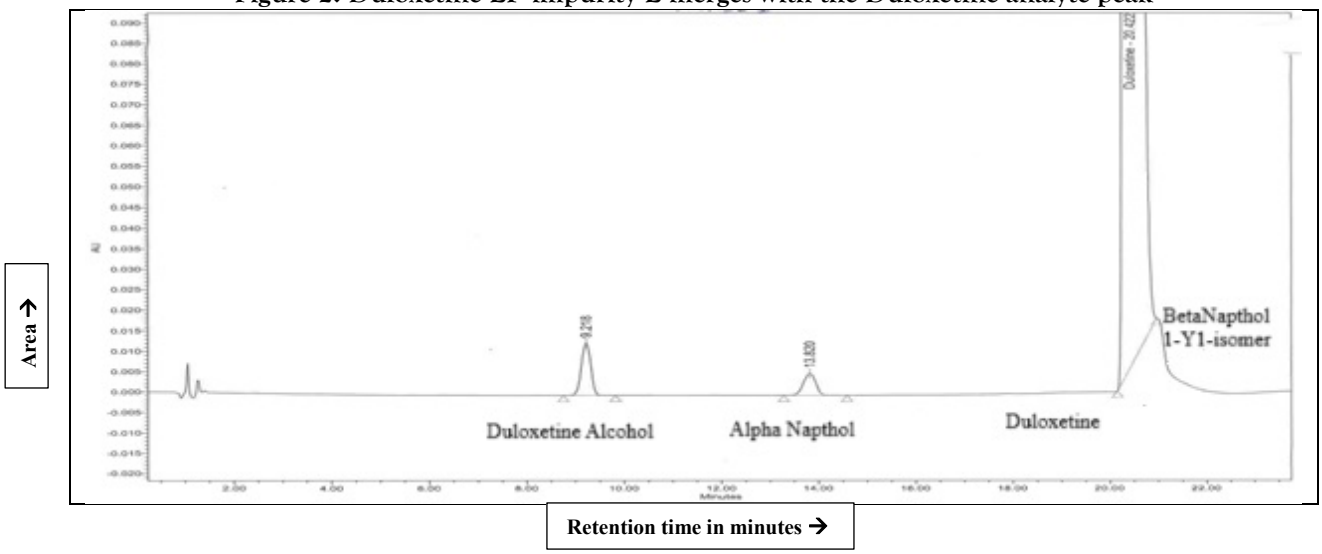
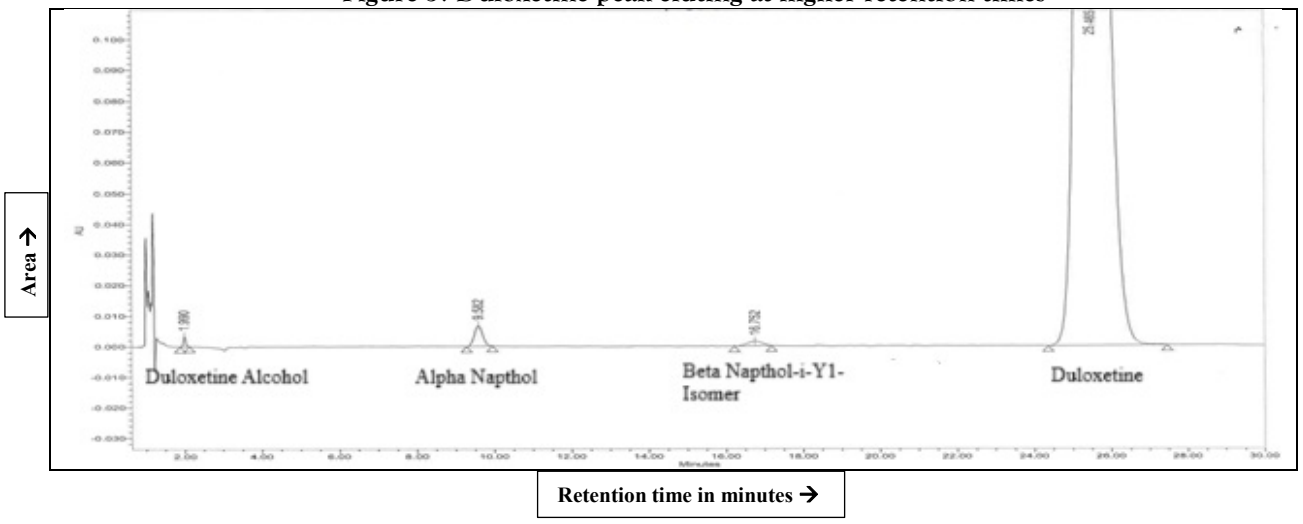
Figure 2: Duloxetine EP impurity E merges with the Duloxetine analyte peak



Further refined the process by altering the mobile phase conditions to a buffer, methanol, and acetonitrile ratio of 70:15:15 v/v/v. (*buffer solution prepared by using 25mM Orthophosphoric adjusted the solution to pH 2.5 with 1N Sodium hydroxide solution (NaOH). Then added 10.3 g of 1-Heptane sulphonic acid sodium salt to the buffer solution, dissolved it and mixed well*), and chromatographic column changed to Hypersil BDS C18,150 x 4.6mm, 5 µm column at flow rate 2.0 mL/min with injection volume 20 µL at detection wavelength 230 nm shows Duloxetine peak eluting at higher retention times.

Figure 3: Duloxetine peak eluting at higher retention times



The above method further optimized by changing the mobile phase ratio to 70:13:17 v/v/v, Buffer, methanol, and isopropyl alcohol, respectively, with the chromatographic conditions such as, Hypersil BDS C18,150 x 4.6 mm,5 µm column, column temperature at 40°C, flow rate 2.0 mL/min, Injection volume 20 µL and detection wavelength 230 nm shows good separated chromatographic profile with shortest run time 30 mins. Duloxetine peak elutes at about 19 mins RT, Duloxetine Alcohol impurity (Duloxetine EP Impurity B), Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E) elutes at retention times about 1.6 min, 7.6 min and 13.1 min at RRT 0.08, 0.38 and 0.66, respectively.

Figure 4: Finalized specimen Chromatogram

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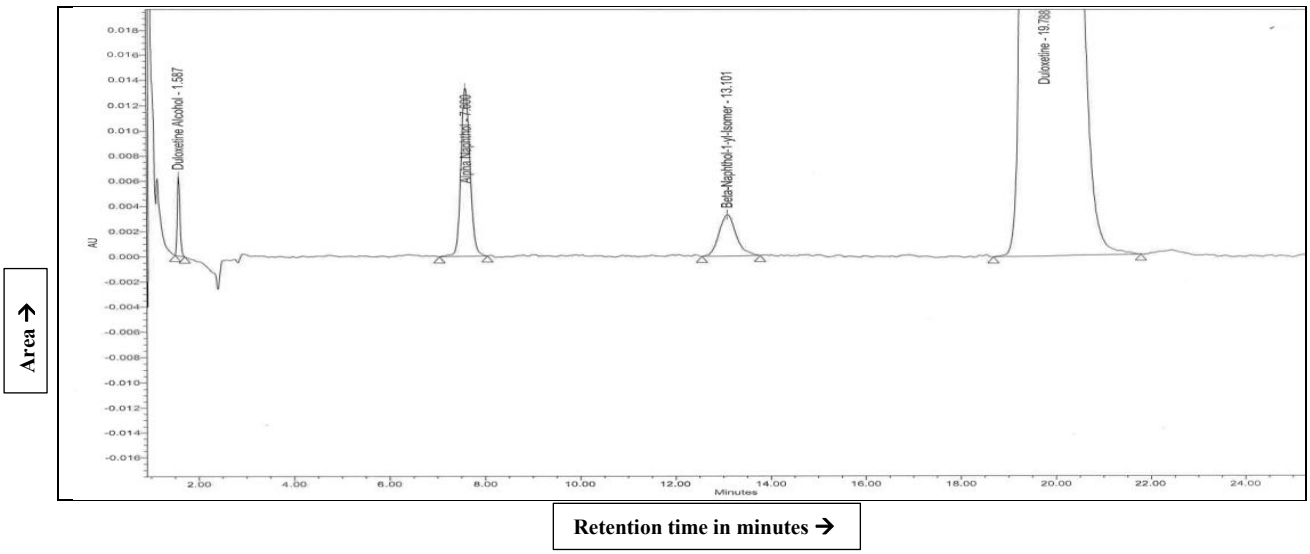

This current method is an isocratic mode with good separation between process impurities and degradation impurities generated during stress study and established the method as 'Stability Indicating Method (SIM).' Hence, this method can be used to Quantify both process related impurities and degradation impurities in finished dosage formulation 'Duloxetine hydrochloride capsules' for routine Quality Control (QC) batch release analysis purpose. In Earlier published methods there is no Isocratic method with SIM established were reported.

2.4.3. Analytical Method Validation

The validation of an analytical technique is to confirm its appropriateness for its intended purpose. Duloxetine hydrochloride related substances by HPLC method were validated as per ICH recommendations[29]. Specificity, System Precision, Method precision, Intermediate precision, Accuracy (Recovery), LOD & LOQ establishment, Precision at LOQ, Recovery at LOQ level, Linearity, and Forced degradation studies (FDS) were validated for the determination of process related impurities and degradation products of Duloxetine hydrochloride by reversed phase liquid chromatography. After finalization of the analytical method, Relative Response Factor (RRF) established using concentration method.

2.4.3.1 Relative Response Factor Establishment (RRF)

Concentration method: prepared impurity solutions and Duloxetine hydrochloride working standard solution at 0.3% level and 0.6% level with respect to test sample concentration and injected in duplicate in finalized analytical method and measured the peak responses. From the obtained chromatographic responses calculated RRF values of impurities with respect to Duloxetine hydrochloride working standard response factor. The Obtained RRF values are captured below table-3.

Table 3: Relative response factor establishment (RRF)

Impurity Name	Chemical Name	RRF
Duloxetine Alcohol impurity (Duloxetine EP Impurity B)	(1S)-3-(Methylamino)-1-(thiophen-2-yl) propan-1-ol	0.34
Duloxetine alpha Naphthol (Duloxetine EP Impurity D)	Naphthalen-1-ol	2.47
Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E)	2-[(1RS)-3-(Methylamino)-1-(thiophen-2-yl) propyl] naphthalen-1-ol	1.07

2.4.3.2 The Method Validation results were summarized in table-4.

Table 4: Results summary of analytical method validation

Validation parameter	Duloxetine EP Impurity B (Duloxetine Alcohol impurity)	Duloxetine EP Impurity D (Duloxetine alpha Naphthol)	Duloxetine EP Impurity E (Beta Napthol-1-yl-isomer impurity)
Specificity			
Individual peak Identified at RT (mins)	1.589	7.608	13.099
Impurity Solution spiked in sample solution at RT (mins)	1.587	7.600	13.101
Blank Interference	No	No	No
Placebo Interference	No	No	No
Precision (%RSD)			
Method Precision (n=6) %RSD	2.06	1.42	1.20
Confidence interval at 95% Level	0.21 to 0.22	0.20 to 0.21	0.22 to 0.23
Intermediate Precision (n=6) %RSD	1.39	1.64	0.40
Confidence interval at 95% Level	0.22 to 0.22	0.20 to 0.22	0.23 to 0.23
Cumulative %RSD (n=12)	2.78	1.72	0.95
LOD & LOQ			
LOD (ppm) / in %	0.277 / 0.028	0.069 / 0.007	0.296 / 0.030
LOD S/N	18.3	8.1	6.8
LOQ (ppm) / in %	0.547 / 0.055	0.122 / 0.012	0.536 / 0.054
LOQ S/N	31.1	11.2	11.7
Precision at LOQ (n=6) in %RSD	3.51	5.33	1.25
Recovery at LOQ (n=3) in %	108.19	111.04	106.83
Linearity			
Linear equation (y=mx+c)	y = 111328x + 1241.9	y = 848600x + 5343.1	y = 365062x - 1762.7
Residual Sum of Squares (R^2)	0.9981	0.9997	0.9980
Accuracy (% Recovery)			
50% Level (n=3)	103.56	107.44	102.84
100% Level (n=3)	104.92	103.18	112.43
150% Level (n=3)	105.36	105.93	110.72
Solution stability (% difference in result from initial solution)			
Day-1	0.001	0.003	0.002
Day-2	-0.005	0.016	0.011

2.4.3.3. System Suitability Test (SST).

System suitability test is the basic requirement for confirming the system performance and obtained results through chromatographic analysis. Every test parameter needs a system suitability test. If system suitability conditions are fulfilled, the column, technique, and HPLC system are acceptable. In the current work, verification of system suitability requirement is ensured for each validation parameter. The system suitability test was ensured by injecting blank injection in the sequence followed by replicate standard injections (6 times) as per the finalized method conditions and system suitability results found to be met with acceptance criteria. For details refer to table 5.

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ISSN: 2229-7359
Vol. 11 No. 15s, 2025
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Table 5: System suitability test results

Compound Name	% RSD	Tailing Factor	Theoretical plates
Duloxetine Hydrochloride DR capsules	6.1	1.0	7118
Acceptance Criteria	NMT: 15.0 %	NMT:2.0	NLT: 2000

2.4.3.4. Specificity

The specificity of the test method was established by injection blank, placebo, individual impurities at specification level (0.2 %) and test sample spiked with impurity solutions at specification level. The acquired chromatograms indicate the absence of peak interference from the blank and placebo at the retention times of both the impurity peaks and the analyte peak in the injected test sample solutions. [figure-5 to 10 for Specimen chromatograms].

Figure 5: Typical Chromatogram of Blank solution injection

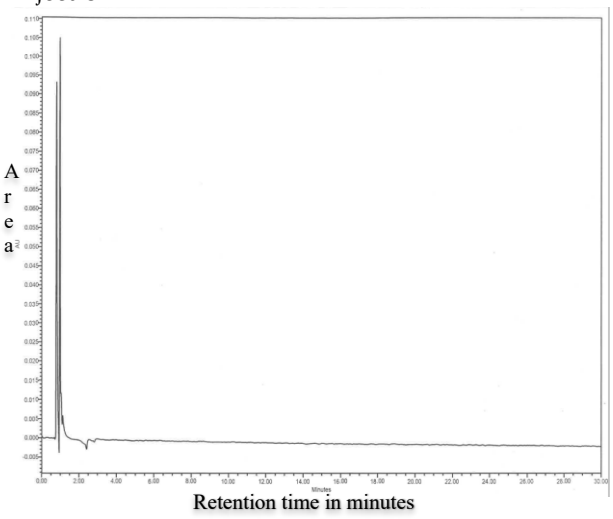


Figure 6: Typical Chromatogram of Placebo solution injection

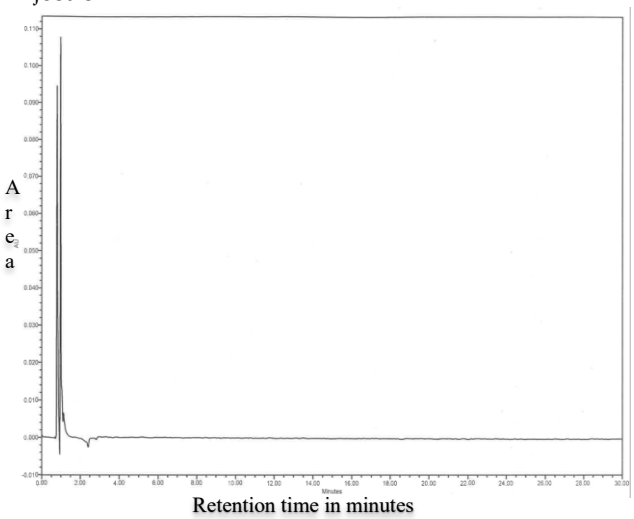


Figure 7: Typical Chromatogram of Duloxetine Alcohol impurity solution injection

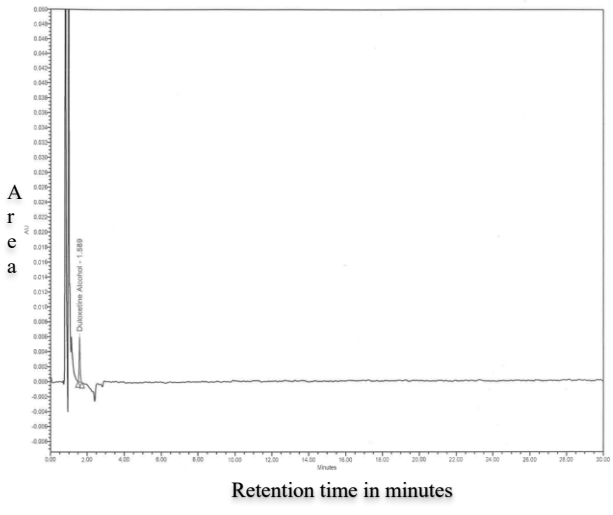


Figure 8: Typical Chromatogram of Duloxetine alpha Naphthol solution injection

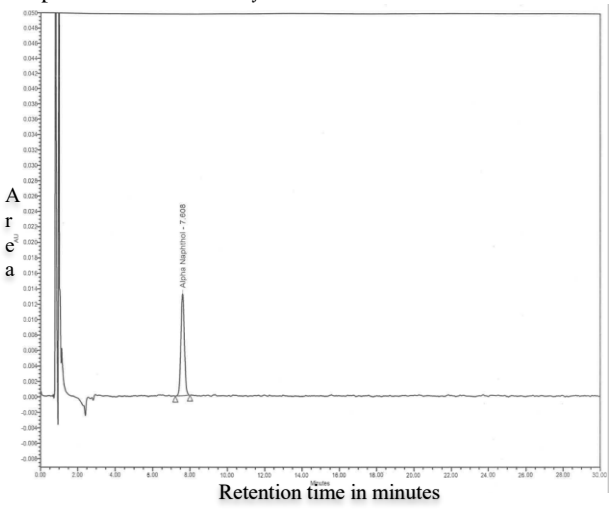


Figure 9: Typical Chromatogram of Beta Napthol-1-yl-isomer impurity solution injection

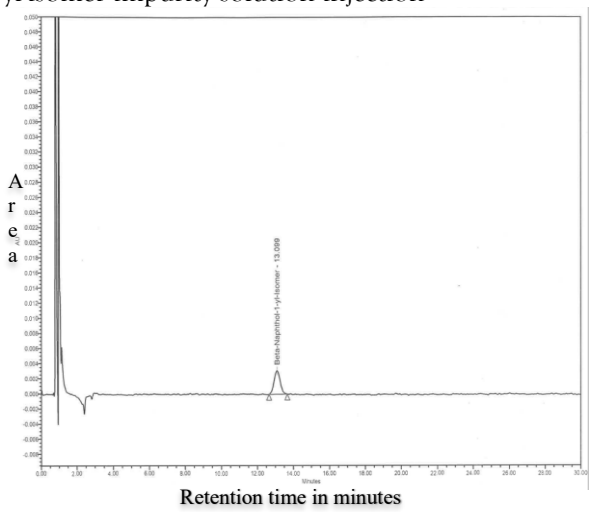
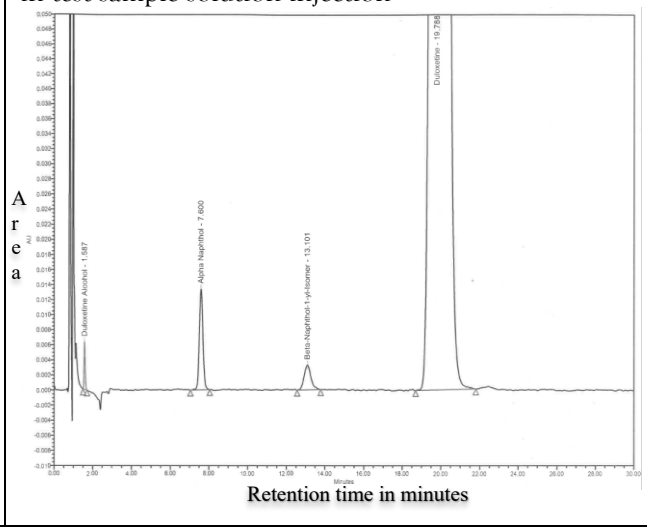


Figure 10: Typical Chromatogram of Impurity spiked in test sample solution injection



2.4.3.5. Precision.

For system precision, the %RSD from six replicate standard injections was found to be 6.1 % Table-5. The obtained individual spiked impurities in test solution from method precision was found to be at specification level (0.2 %) and the %RSD of Duloxetine EP Impurity B, Duloxetine EP Impurity D & Duloxetine EP Impurity E was 2.06 %,1.42 % and 1.20 % respectively. Similarly, the % RSD of individual spiked impurities in test solution from Intermediate method precision was found to be at specification level (0.2 %) and the %RSD of Duloxetine EP Impurity B, Duloxetine EP Impurity D & Duloxetine EP Impurity E was found to be 1.39 %, 1.64 % & 0.40 % respectively. The %RSD findings indicate strong concordance among the individual preparations, demonstrating that the method employed is precise. The calculated 95 % confidence intervals during method precision and intermediate precision details are captured in Table-4.

2.4.3.6. Limits of detection and quantification.

The limits of detection (LOD) and quantification (LOQ) were determined using the formulas specified in the ICH guidelines: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ represents the standard deviation of the response and S denotes the slope of the calibration curve. The found signal-to-noise ratio is greater than 3 for LOD value and greater than 10 for LOQ value. The concentration of LOD values were established at 0.277 ppm, 0.069 ppm and 0.296 ppm for Duloxetine Alcohol impurity (Duloxetine EP Impurity B), Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E) respectively. The concentration of LOQ values were established at 0.547 ppm, 0.122 ppm and 0.536 ppm for Duloxetine Alcohol impurity (Duloxetine EP Impurity B), Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E) respectively. The %RSD of impurity precision at LOQ level found to be 3.51%, 5.33% and 1.25% for Duloxetine Alcohol impurity (Duloxetine EP Impurity B), Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E) respectively. The Recovery at LOQ level found to be 108.19%,110.4% and 106.83% for Duloxetine Alcohol impurity (Duloxetine EP Impurity B), Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E) respectively Table-4.

2.4.3.6. Linearity and range.

The linearity of the detector response was established for all three impurities and Duloxetine hydrochloride across the range from the limit of quantification (LOQ) to 150%. This includes the following test concentration levels: LOQ, 50%, 75%, 100%, 125%, and 150%. The linearity study was conducted from 0.547 ppm to 3 ppm for Duloxetine Alcohol Impurity (Duloxetine EP Impurity B), 0.122 ppm to 3 ppm for Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D) and 0.536 ppm to 3 ppm for Beta Napthol-1-yl-isomer impurity

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(Duloxetine EP Impurity E). The calibration curve was drawn by concentration of Linearity solutions in X-axis (in ppm) and area responses on Y-axis (AU) using Linear regression analysis. [figure 11 to 14 for linearity graphs]. The areas of the impurity peaks demonstrated a linear relationship across the concentration range, with correlation coefficients (R^2) exceeding 0.99 Table-4. The established linear relationship between solution concentration and area responses indicates that the method is capable of quantifying impurities within this defined linear range.

Figure 11: Linearity graph of Duloxetine alcohol Impurity (Duloxetine EP Impurity B)

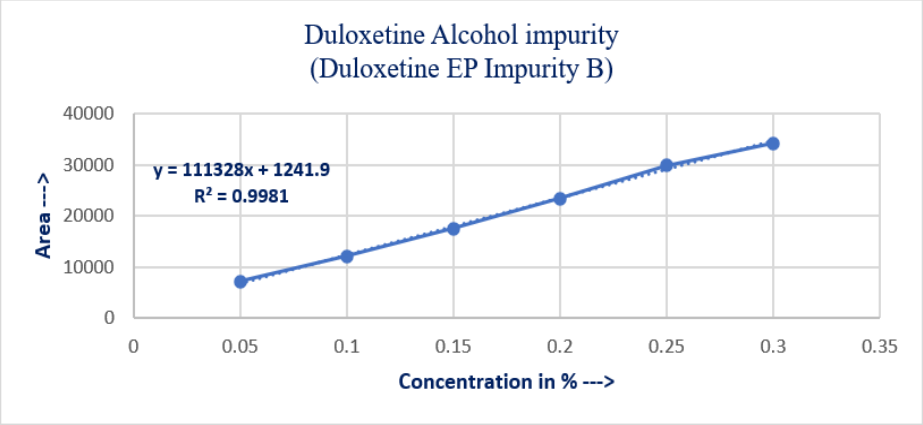


Figure 12: Linearity graph of Duloxetine alpha Naphthol Impurity (Duloxetine EP Impurity D)

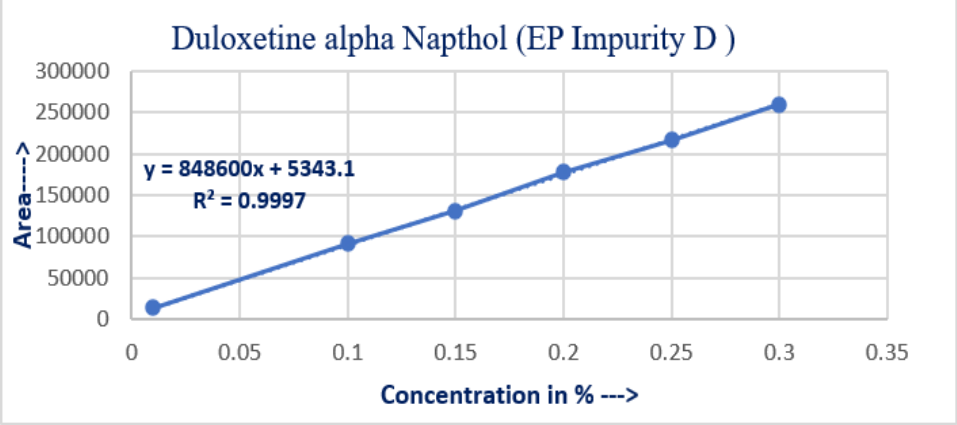


Figure 13: Linearity graph of Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E)

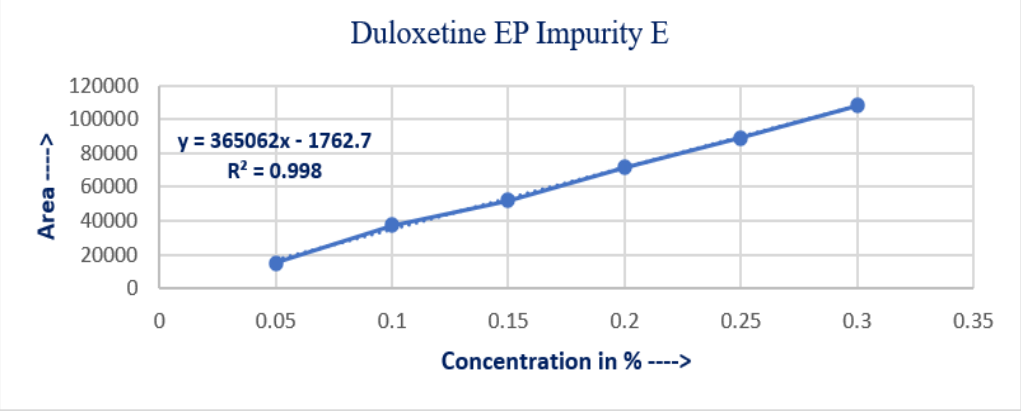
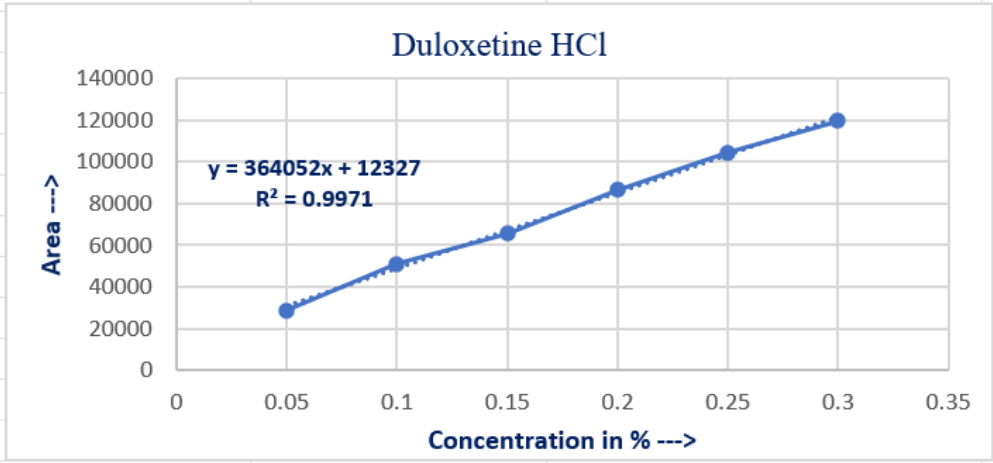


Figure 14: Linearity graph of Duloxetine Hydrochloride



2.4.3.7. Accuracy (Recovery)

The recovery study was conducted at 50 %,100 % and 150 % levels by spiking impurities mixture at 0.2 % level on test sample solution with respect to test concentration. The average recovery obtained for all three impurities at three distinct levels ranged from 102.84% to 112.43% Table-4.

2.4.3.8 Stability of Solutions.

The stability of sample solutions was established up to 48 h (2days) at room temperature and found to be stable up to 48 h at room temperature Table-4.

2.4.3.9. Specificity (Forced Degradation Studies)

In the current forced degradation study conducted stress studies using acid hydrolysis (used 0.1N HCl, kept aside on workbench at room temperature for 6 days), Base hydrolysis (used 0.1N NaOH, kept on water bath at 60°C for 24 h), Oxidative stress study (used 3% H_2O_2, kept aside on work bench at room temperature for 3 h), Humidity stress study (exposed sample and placebo to 75% Relative Humidity (RH) for 24 h),Thermal stress study (at 60°C for 24 h), and Photolytic stress study (200-Watt hours/m^2 for UV light and 1.2 million lux hours for Visible light).Significant degradation was noted in the acid stress study, with a net degradation percentage of 12.5. Nevertheless, the Mass Balance for all stressed samples was determined to be around 100%. The % Net degradation was found to be high in acid stress sample and in other stress conditions no significant degradation had happened (Table-6). In all stress condition samples, found "purity angle < purity threshold angle", indicates there is no co-elution along with the analyte peak. [Figure-15 to 28 for sample chromatograms (as such & zoomed)] and [Figure-29 to 34 for peak purity plots].

Table 6: Forced Degradation study results and Mass Balance of all stressed samples

Stress Condition	Assay (in %)	Total impurities (in %)	Net Degradation (in %)	Mass balance (Assay + Total impurities)
Control Sample (Unstressed)	99.5	0.05	--	--
Acid Stress Study_0.1N HCl_RT_24h	87.03	13.5	12.5	101.04
Base Stress Study_0.1N NaOH_RT_24h	98.20	1.90	1.3	100.60
Oxidative Stress Study_3%_RT_3h	99.93	0.05	-0.4	100.48
Humidity Study_75%RH_24h	99.5	0.06	0.0	100.06
Thermal Stress Study_60°C for 10 days	99.95	0.05	-0.5	100.50
Photolytic Stress study_200-Watt hours/m2 (UV),1.2 million lux hours (Visible)	99.94	0.07	-0.4	100.51

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Figure 15: Specimen chromatogram of Control Sample

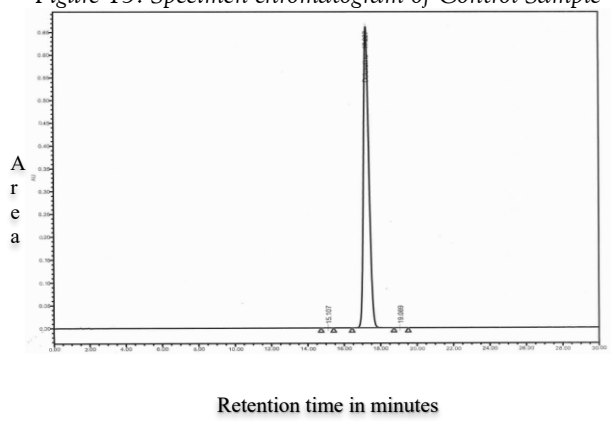

Figure 16: Specimen chromatogram of Control Sample (Zoomed)

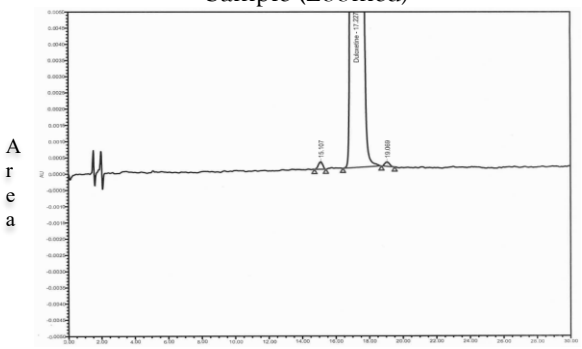

Figure 17: Specimen chromatogram of Acid hydrolysis Stress Sample

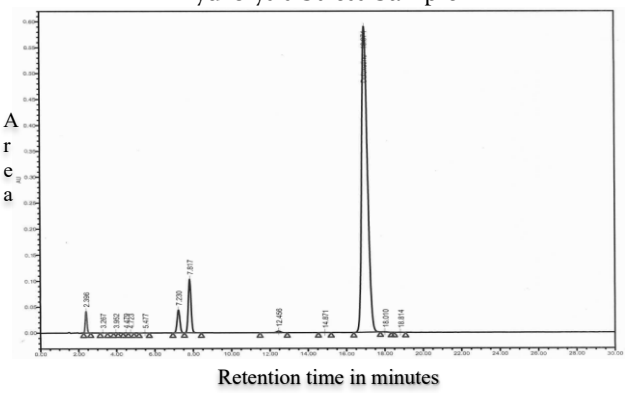

Figure 18: Specimen chromatogram of Acid hydrolysis Stress Sample (Zoomed)

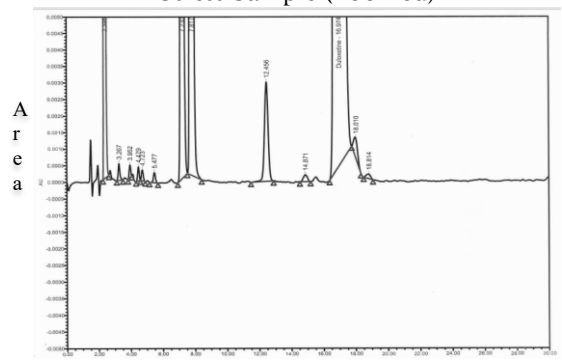

Figure 19: Specimen chromatogram of Base hydrolysis Stress Sample

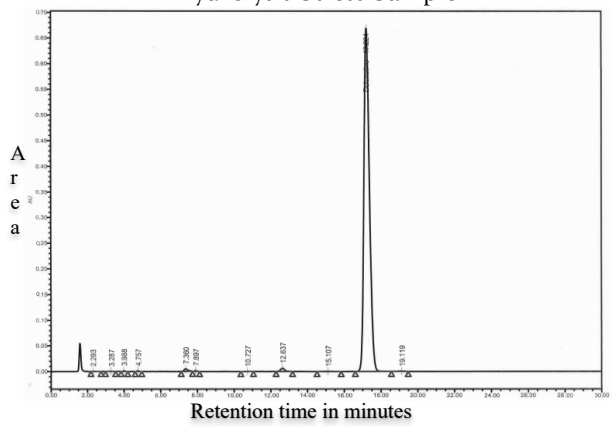

Figure 20: Specimen chromatogram of Base hydrolysis stress Sample (Zoomed)

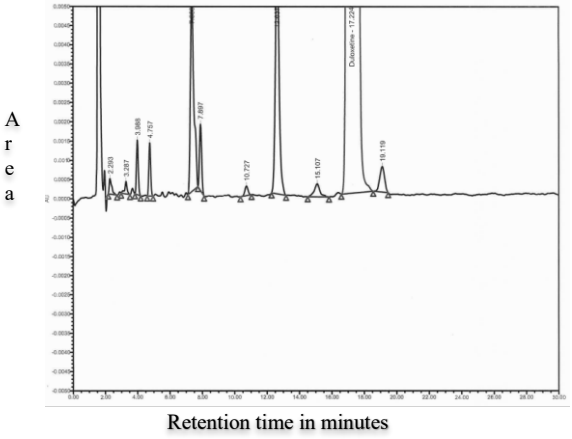

Figure 21: Specimen chromatogram of Oxidative Stress Sample

Figure 22: Specimen chromatogram of Oxidative Stress Sample (Zoomed)

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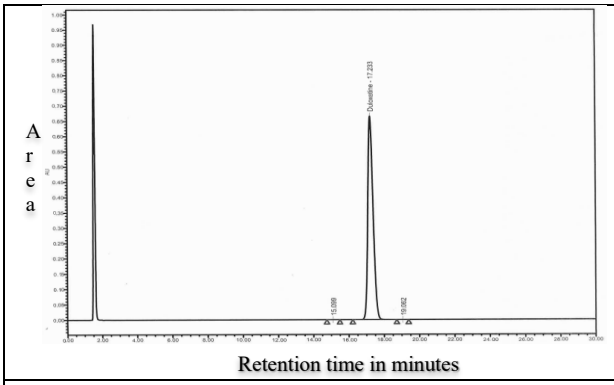

Figure 23: Specimen chromatogram of Humidity Stress Sample

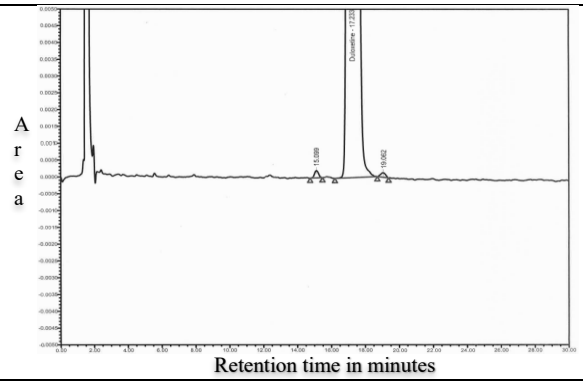

Figure 24: Specimen chromatogram of Humidity Stress Sample (Zoomed)

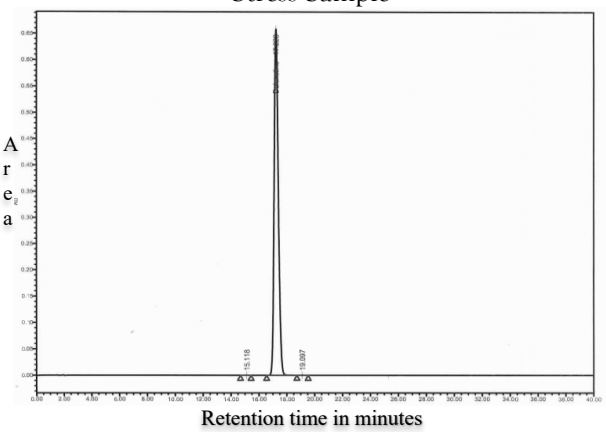

Figure 25: Specimen chromatogram of Thermal Stress Sample

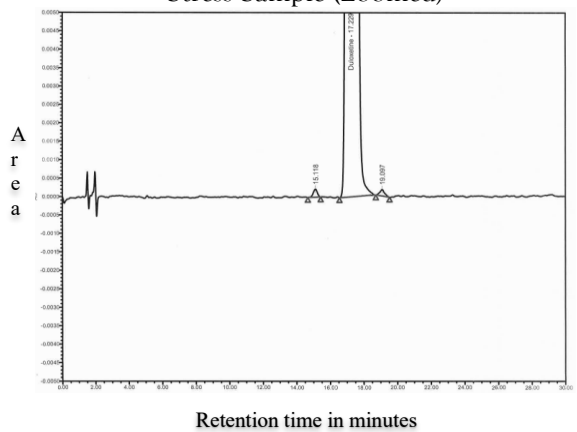

Figure 26: Specimen chromatogram of Thermal Stress Sample (Zoomed)

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Figure 27: Specimen chromatogram of Photolytic Stress Sample

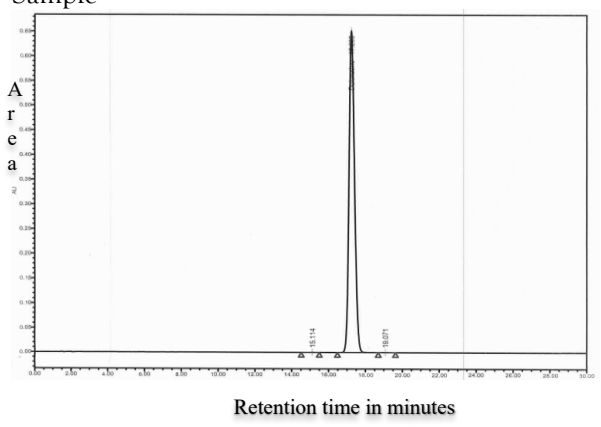
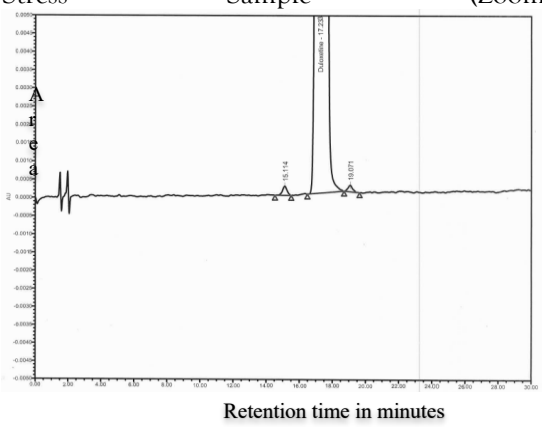


Figure 28: Specimen chromatogram of Photolytic Stress Sample (Zoomed)



Peak Purity plots of all stressed samples:

Figure 29: Peak purity plot in Acid Hydrolysis stress sample

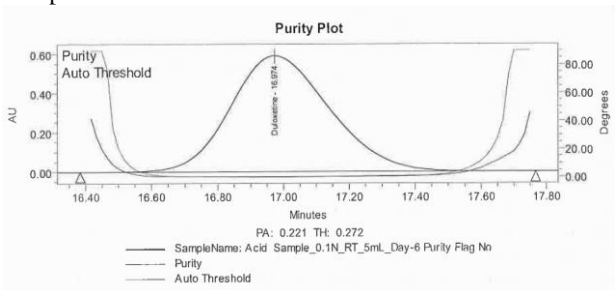


Figure 30: Peak purity plot in Base Hydrolysis stress sample

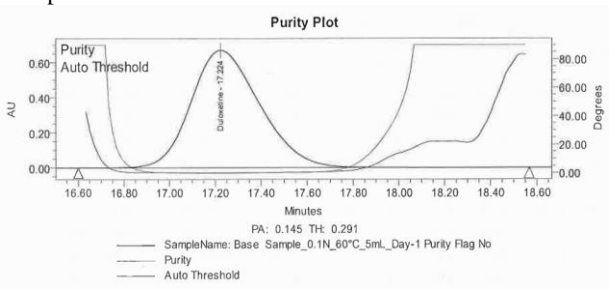


Figure 31: Peak purity plot in Oxidative stress sample

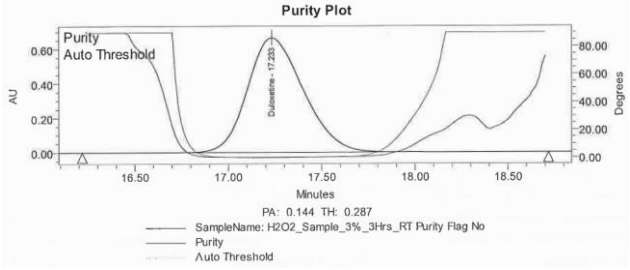


Figure 32: Peak purity plot in Humidity stress sample

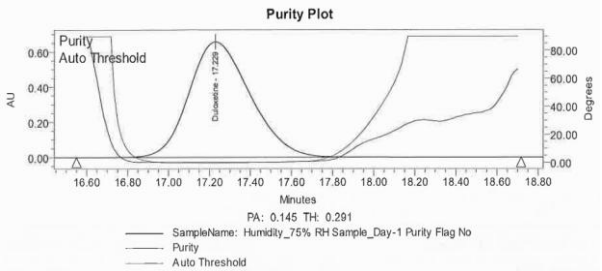


Figure 33: Peak purity plot in Thermal stress sample

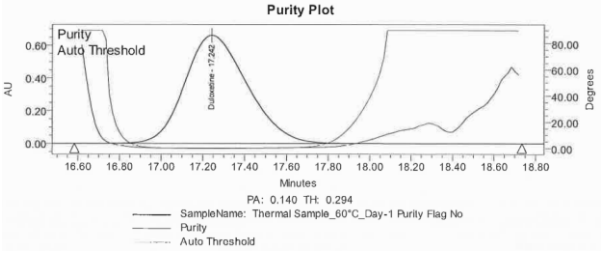
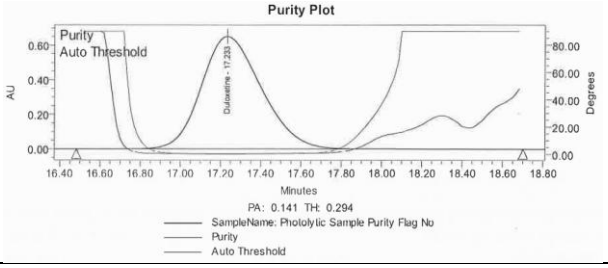


Figure 34: Peak purity plot in Photolytic stress sample



2.4.3.9. Batch Analysis

The developed reverse phase chromatography method was used on routine quality control batch release of Duloxetine Hydrochloride DR Capsules 60mg. Three batches of finished products were chosen for batch analysis, and samples were prepared according to the sample preparation procedure and quantified against

diluted standard average area from replicate standard injections by using RRF values. All test sample findings were below the Limit of Detection (BLOD). The collected findings are shown in Table 7.

Table 7: Batch analysis results

Impurity Name	Batch-1	Batch-2	Batch-3
Duloxetine Alcohol impurity (Duloxetine EP Impurity B)	BLOD	BLOD	BLOD
Duloxetine alpha Naphthol (Duloxetine EP Impurity D)	BLOD	BLOD	BLOD
Beta Napthol-1-yl-isomer impurity (Duloxetine EP Impurity E)	BLOD	BLOD	BLOD
Any individual unspecified degradation product	BLOD	BLOD	BLOD
Total Impurities	BLOD	BLOD	BLOD

3. Conclusions

The developed isocratic reversed phase chromatographic method for the determination of Duloxetine Hydrochloride DR capsules, process related, and degradation impurities are found to be specific, precise, accurate, linear, rugged and robust. The demonstration of forced degradation study reveals that the selected impurities and potential degradation impurities were eluting within 30 mins of run time and meeting its mass balance criteria in all stressed samples, which indicates that the analytical method can detect and quantify all impurities (both process and degradation impurities) with good resolution and precise quantification. The current methodology able to quantify degradation impurities (Duloxetine alpha Naphthol) at 0.012% level and process related impurities (Duloxetine Alcohol impurity & Beta Napthol-1-yl-isomer impurity) at 0.05% level. Method has the capability to detect degradation impurities (Duloxetine alpha Naphthol) at 0.007% level and process related impurities (Duloxetine Alcohol impurity & Beta Napthol-1-yl-isomer impurity) at 0.003% level. The application of analytical method to routine Quality control batch sample analysis reveals that the method is suitable for routine usage. Hence, the current isocratic method with run time (30 mins) can be used for determining the Duloxetine impurities for Quantification of both process and degradation impurities in routine analysis.

4.Consent for publication:

The authors declare no conflict of interest.

5.Competing interests:

The authors declare that they have no competing interests.

6. Acknowledgements:

Ramesh Nethala is grateful to Lucid Laboratories, Hyderabad, India, for providing research facility to conduct research.

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