International Journal of Research in Pharmacy and Pharmaceutical Sciences
ISSN: 2455-698X; Impact Factor: RJIF 5.22
Received: 02-04-2020; Accepted: 20-05-2020; Published: 06-05-2020
www.pharmacyjournal.in
Volume 5; Issue 3; 2020; Page No. 01-05

Development and evaluation of novel estimation techniques and validation protocol for duloxetine as antidepressant drug and their formulation

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Abstract
The present study indicates a simple, accurate and precise reverse phase high performance liquid chromatography was developed and validated for the estimation of duloxetine in pharmaceutical dosage form. The mobile phase used was Phosphate buffer: acetonitrile 30:70v/v (pH: 4.2). The specification of the chromatographic system is waters C18, 5µm, 150mm x4.6mm, flow rate 0.8ml/min, detection 225nm, and injection volume 20µl and run time 6 min. The retention times found to be 2.26 min. The linearity curve was in the range of 30-70 ppm. The recoveries found to be 99.25 to 100.43%. The proposed method was validated and successfully applied to the estimation of duloxetine and its dosage forms.

Keywords: RP-HPLC, duloxetine, analytical method development, anti-depressant activity

Introduction

Duloxetine hydrochloride is chemically, methyl-[(3S)-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl) propyl] amine. The Molecular formula is $C_{18}H_{19}NOS$. It is a potent inhibitor of serotonin and norepinephrine reuptake, and thus used for major depressive disorders [1, 3]. Duloxetine increasing serotonin and norepinephrine concentrations in Onuf's nucleus it enhances the glutamatergic activation of the pudendal motor nerve which innervates the external urethral sphinter. [4, 6] This enhanced signaling allows for stronger contraction. Increased contraction of this sphincter increases the pressure needed to produce an incontinence episode in stress urinary incontinence. Action at the dorsal horn of the spinal cord allows duloxetine to strengthen the serotonergic and adrenergic pathways involved in descending inhibition of pain [7]. Duloxetine is a potent inhibitor of neuronal serotonin and norepinephrine reuptake and a less potent inhibitor of dopamine reuptake [8]. Duloxetine has no significant affinity for dopaminergic, adrenergic, cholinergic, histaminergic, opioid, glutamate, and GABA receptors. Over 90% bound to plasma proteins, primarily albumin and α1 acid-glycoprotein. Duloxetine is extensively metabolized primarily by CYP1A2 and CYP2D6 with the former has more contribution. It's hydrolysis takes place at the 4, 5, or 6 positions on the naphthalene ring. The 4-hydroxy metabolite continued directly to a glucuronide conjugate while the 5 and 6-hydroxy metabolites passed through a catechol and 5-hydroxy, 6-methoxy intermediate before undergoing to glucuronide or sulfate conjugation. About 70% of drug is excreted in the urine mainly as conjugated metabolites. Another 20% is present in the feces as the parent drug, 4-hydroxy metabolite, and an uncharacterized metabolite [9]. Furthermore, it has an effect on pain in urinary incontinence [10, 11] independent of its effect on depression. In adults it is used to treat fibromyalgia, chronic muscle or joint pain. It is used to treat pain caused by nerve damage in adults with diabetes. Duloxetine is approved for a variety of indications includes treatment of neuropathic pain, Generalized Anxiety disorder, Osteoarthritis, and stress incontinence. Duloxetine is under investigation for the treatment of pain in cancer, surgery, and more [12].

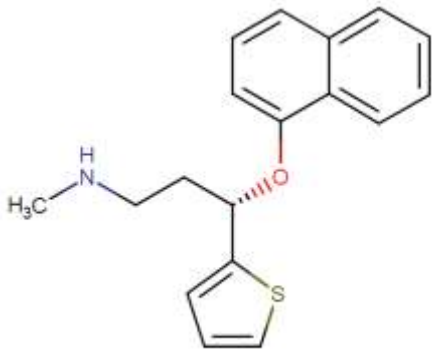

Fig 1: Structure of duloxetine

The literature survey reveals spectrophotometric, RP-HPLC, and HPTLC methods for duloxetine either individually or in combination with other formulations. The literature review does not show any stability indicating RP-HPLC [13] method for quantification of duloxetine. The Navneet Kumar *et al*. developed and validated the new UPLC [14] for the determination of duloxetine hydrochloride residues on pharmaceutical manufacturing equipment surfaces. Sk. Zakir Hussain *et al*, developed a delayed release pellets dosage form of duloxetine hydrochloride and studied the in vitro dissolution studies [15] and many others worked to develop the RP-HPLC method of duloxetine [16, 17]. The present research work was aimed to develop a single, simple, fast, rapid suitable stability-indicating RP-HPLC method for the determination of duloxetine. The developed method was validated with respect to specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy and robustness. The developed method was validated with respect to specificity, limit of detection

(LOD), limit of quantitation (LOQ), linearity, precision, accuracy and robustness. Forced degradation studies were performed on the drug product solution to show the stability-indicating nature of the method and to ensure its compliance in accordance with International Conference on Harmonization (ICH) guidelines [18].

Materials and Methods

Chemicals and reagents

Samples of Duloxetine pure drugs were received from; Lupin Pharmaceuticals, Inc. (Mumbai, India). HPLC-grade acetonitrile was purchased from Loba Chem; Mumbai. Ortho-phosphoric acid was purchased from Sd fine-Chem ltd; Mumbai. L.R grade Potassium dihydrogen orthophosphate was purchased from Sd fine-Chem ltd; Mumbai. HPLC grade water was prepared by using a Millipore Milli-Q plus purification system.

HPLC Instrumentation & Conditions

The HPLC system employed was HITACHI L2130 with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400),

Standard & sample preparation for UV-spectrophotometer analysis

25 mg of Duloxetine standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Duloxetine, so that the same wave number can be utilized in HPLC UV detector for estimating the Duloxetine. While scanning the Duloxetine solution we observed two maxima's at 230 & 256 nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

Standard Solution Preparation

25 mg of Duloxetine standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase. The solution was mixed well and filtered through 0.45µm filter.

Sample Solution Preparation

Twenty capsules were taken and the I.P. method was followed to determine the average weight. Above weighed capsules were finally powdered and triturated well. A quantity of powder equivalent to 100 mg of drugs were transferred to 100 ml volumetric flask, and 70 ml of diluents was added and solution was sonicated for 15 minutes, there after volume was made up to 100 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with diluents. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

Preparation of Phosphate buffer and Mobile phase

The mobile phase used in this analysis consists of a mixture of Buffer (0.05 M potassium dihydrogen phosphate & pH adjusted to 4.2 with Orthophosphoric acid) and Acetonitrile in a ratio of 30:70. 700 ml of this buffer solution was added and properly mixed with 300 ml of acetonitrile and a homogenous solution is achieved. This mobile phase was filled and sonicated for 5 minutes before using in the experiment.

Diluent Preparation

Mobile phase as diluent.

Result and Discussion

Initialization of the instrument

The HPLC instrument was switched on. The column was washed with HPLC water for 45min. The column was then saturated with mobile phase for 45min. The mobile phase was run to find the peaks. After 20 minutes the standard drug solution was injected in HPLC.

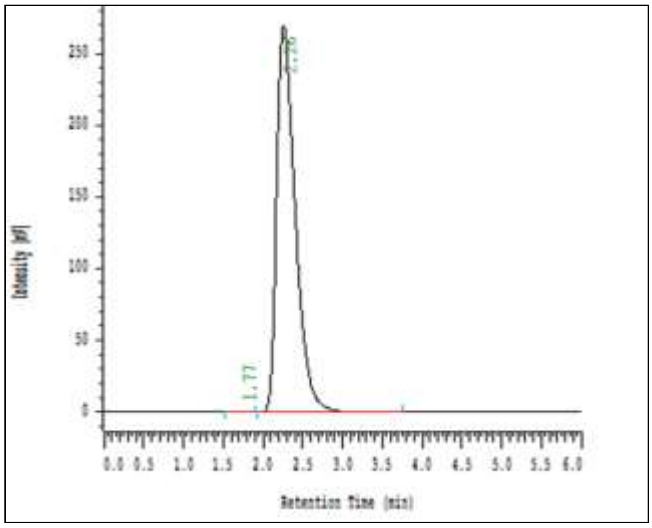

Fig 2: HPLC spectrum of Duloxetine (50 ppm) in optimized conditions (RT 2.26 min.)

Table 1: Optimized Chromatographic Conditions

1	Column	C18 Develosil ODS HG-5 RP 150mm x4.6mm 5µm particle size
2	Mobile Phase	Phosphate buffer: acetonitrile 30:70 pH:4.2
3	Flow Rate	0.8ml/minute
4	Wave length	225nm
5	Injection volume	20 µl
6	Run time	6 minutes
7	Column temperature	Ambient

Table 2: Different Trials for Chromatographic Conditions

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Waters C18, 5µm, 25cmx4.6mm i.d.	phosphate buffer: acetonitrile Ratio: 70:30 pH: 3.5	1 ml/min	256 nm	Low response	Method rejected
Waters C18, 5µm, 25cmx4.6mm i.d.	phosphate buffer: acetonitrile Ratio: 70:30	1 ml/min	256 nm	low response	Method rejected

	pH:4				
Waters C18, 5µm, 25cmx4.6mm i.d.	water: acetonitrile 50:50	1.3 ml/min	256 nm	Broad Peak	Method rejected
Waters C18, 5µm, 25cmx4.6mm i.d.	phosphate buffer: acetonitrile 60:40 pH:4.2	1.3 ml/ min	230 nm	Tailing peak	Method rejected
Waters C18, 5µm, 25cmx4.6mm i.d.	phosphate buffer: acetonitrile 30:70 pH:4.2	0.8 ml/min	225nm	Good response	Method accepted

Method Validation

Accuracy: Recovery study

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Duloxetine were taken and added to the pre-analyzed formulation of concentration 50µg/ml. From that percentage recovery values were calculated.

Table 3: Accuracy Readings For 80%of Duloxetine

concentration in ppm	Rt of Duloxetine	Peak area of Duloxetine	recovery
50 +40	2.21	8007244	99.33925
50 +40	2.22	8056564	100.7428
50 +40	2.22	8072938	101.2087
Avg	0.005774	34196.17891	0.973128
Standard deviation	2.216667	8045582	100.4302
%RSD	0.260459	0.425030519	0.968959

Table 4: Accuracy Readings For 100% of Duloxetine

concentration in ppm	Rt of Duloxetine	Peak area of Duloxetine	Recovery
50 +50	2.25	8807444	98.48498
50 +50	2.24	8890650	100.3947
50 +50	2.25	8824707	98.88119
Avg	0.005774	43912.27781	1.007847
Standard deviation	2.246667	8840933.667	99.25361
%RSD	0.256981	0.496692764	1.015426

Table 5: Accuracy readings for 120% of Duloxetine

Concentration in ppm	Rt of Duloxetine	Peak area of Duloxetine	Recovery
50+60	2.24	9994211	100.5212
50+60	2.23	9951108	99.73021
50+60	2.24	9992432	100.4885
Avg	0.005774	24388.20215	0.44754
Standard deviation	2.236667	9979250.333	100.2466
%RSD	0.25813	0.244389121	0.446439

System suitability studies

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 50 µg/ml.

Table 6: System Suitability Studies of Duloxetine

Sr.no.	Concentration	Retention time	Peak area	Theoretical plates	Tailing factor
1	50	2.26	4371369	6320	1.01
2	50	2.26	4327048	6389	0.98
3	50	2.26	4372696	6366	0.99
4	50	2.26	4283857	6312	0.99
5	50	2.26	4340455	6353	0.96
6	50	2.26	4329302	6308	0.97
Avg		2.26	4339085	6341.333	0.98333
SD		0	36636.23285	32.99495	0.017512
%RSD		0	0.844330841	0.520316	1.780871

Table 7: Acceptance criteria and result

Sr. No	Parameter	Limit	Result
1	Resolution	Rs > 2	3.15
2	Tailing factor	T ≤ 2	Duloxetine=1.78

Repeatability

The precision of each method was ascertained separately from the peak areas obtained by actual determination of five replicates of a fixed concentration of Duloxetine. The percent relative standard deviations were calculated for Duloxetine.

Table 8: Repeatability Readings For 50 ppm 5 injections

concentration of Duloxetine in ppm	Rt of Duloxetine	Peak area of Duloxetine
50	2.21	4371369
50	2.21	4327048
50	2.21	4372696
50	2.21	4283857
50	2.21	4340455
AVG	2.21	4339085
S.D.	0	36636.23285
%RSD	0	0.844330841

Intra-assay & inter-assay

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Sertraline revealed that the proposed method is precise. The results were shown in table-5

Table 9: Results of intra-assay & inter-assay

Conc. Of Duloxetine (µg/ml)	Observed Conc. Of Duloxetine (µg/ml) by the proposed method			
	Intra-Day Assay		Inter-Day Assay	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
40	2.21	1.6	2.22	1.66
50	2.26	0.90	2.23	1.31
60	2.21	1.18	2.23	1.33

Linearity & Range

Linearity indicates the ability of analytical procedures to produce results that are directly proportional to the concentration of analyte in the given sample. The results shown in table-6

Linearity

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 30, 40, 50, 60 and 70µg/ml. The prepared solutions were filtered through whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table 10: Linearity Readings for Duloxetine

Concentration of duloxetine in ppm	Peak area of duloxetine
0	0
30	2789320
40	3508004
50	4371369
60	5304243
70	6090165

Calibration Curve
Linearity Plot Information

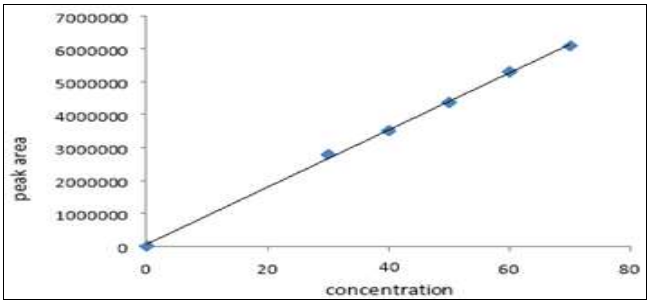

Fig 3: Calibration curve of duloxetine

Procedure
Each level was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

Acceptance Criteria
Correlation coefficient should be not less than 0.999.

Ruggedness

Table 11: Ruggedness study readings by analyst 1

Sr. no	Injection number	Duloxetine	
		Area	Retention time
1	50	2.21	4356688
2	50	2.21	4388290
	Avg	2.21	4372489
	S.D.	0	15801
	%RSD	0	0.361373

Table 12: Ruggedness study readings by analyst 2

Sr. no	Injection number	Duloxetine	
		Area	Retention time
1	50	2.22	4284939
2	50	2.22	4283030
	Avg	2.22	4283984.5
	S.D.	0	954.5
	%RSD		0.02228066

Robustness
To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of wavelength (235 and 239 nm) and mobile phase flow rate by 0.1 ml/min (0.9 and 1.1ml/min) had no significant effect on the retention time and chromatographic response of the 50 µg/ml solution, indicating that the method was robust.

Table 13: Robustness study Observation for 0.9ml/min

Sr. no	Peak area for Duloxetine	Rt for Duloxetine
1	4388232	2.3
2	4371891	2.3
Avg	2923893	2.3
SD	11554.83	0
%RSD	0.395187	0

Table 14: Robustness study Observation for 1.1 ml/min

Sr. no.	Peak area for Duloxetine	Rt for Duloxetine
1	4284939	2.11
2	4283030	2.11
Avg	4283984.5	2.11
SD	954.5	0
%RSD	0.02228066	0

Table 15: Robustness study observation at 235 nm

Sr no	Peak area for Duloxetine	Retention time
1	4471891	2.26
2	4483030	2.26
Avg	4477460.5	2.26
SD	5569.5	0
%RSD	0.124389707	0

Table 16: Robustness study observation at 239 nm

Sr no	Peak area for Duloxetine	Retention time
1	4261891	2.26
2	4161892	2.26
Avg	4211891.5	2.26
SD	49999.5	0
%RSD	1.187103229	0

LOD & LOQ
The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 1.39 & 4.17 µg/ml respectively.

Assay of Duloxetine in Dosage Form
Twenty capsules were taken and the I.P. method was followed to determine the average weight. Above weighed capsules were finally powdered and triturated well. A quantity of powder equivalent to 100 mg of drugs were transferred to 100 ml volumetric flask, and 70 ml of diluents was added and solution was sonicated for 15 minutes, there after volume was made up to 100 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with diluents. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.
The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded.
The data is shown in following table.

Table 16: Assay of duloxetine

Brand name of capsule	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	% Assay
Cymbalta	30.0mg	31.2(±0.498)	103.4506

The amount of drugs in capsules was found to be 31.2 (± 0.498) mg/tab for Duloxetine and % assay was 103.4506.

Conclusion

In this research, a sensitive, specific, selective, accurate, robust, validated and well-defined RP-HPLC method developed for determination of Duloxetine. In this developed method sample preparation is simple, economical, specific, accurate and precise for the given drug molecule and the analysis time is very short and to our present knowledge, no attempts have yet been made to develop and validate the method for the drug and its formulation. The developed RP-HPLC method was validated as per ICH guidelines. Hence this proposed method can be used conveniently for the routine drug analysis in the bulk and its formulations.

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