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RESEARCH ARTICLE

REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY(RP-HPLC) METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF SILDENAFIL CITRATE AND DULOXETINE HYDROCHLORIDE IN COMBINE DOSAGE FORM

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ABSTRACT

Sildenafil citrate (SIL) and Duloxetine (DULO) is a combination, which is a serotonin-norepinephrine reuptake inhibitor (SNRI). The primary goal of the endeavor is to develop and evaluate a stability-indicating reverse-phase liquid chromatography (RP-HPLC) technique for determining Sildenafil citrate and Duloxetine hydrochloride in the combined dosage form. Estimation of Sildenafil citrate and Duloxetine hydrochloride in pharmaceutical dosage form was carried out using Thermoscientific C18BDS- (25 cm x 4.6 mm x 5 mm). The mobile phase used consisted of Acetonitrile: Phosphate buffer (50:50%v/v), and the pH of the mobile phase was adjusted to 4.0 using 0.5%v/v Orthophosphoric acid. The flow rate was 1.0mL/min. The UV detection was done at 290nm. The injection volume was 20µl. Forced degradation studies including hydrolytic (acidic, basic, and neutral), oxidative, and thermal, have been carried out according to ICH Q1A (R2) guidelines. Potential degradation was found in acidic, oxidative, and basic environments. The development of the method was validated according to ICH guidelines, and the findings were deemed acceptable. The devised stability-indicating technique effectively evaluated sildenafil citrate and duloxetine in the pharmaceutical tablet dosage form in the presence of a

degradation product, with test results showing satisfactory recovery.

Keywords: Sildenafil citrate, Duloxetine hydrochloride, Stability indicating, RP-LC, Forced degradation, Method validation.

INTRODUCTION

Sildenafil citrate5-{2-Ethoxy-5-[(4-methylpiperazin-1-yl)sulfonyl]phenyl}-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one [1]. Duloxetine(methyl[(3S)-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propyl]amine)[2]. a serotonin-norepinephrinereuptake inhibitor(SNRI)[3-4], Sildenafil Citrate and Duloxetine HCl active pharmaceutical ingredient(API)is not official in any Pharmacopoeia.Several HPLC (High-performance liquid chromatography) and UV analytical methods have been reported [5-22], however, there is no method available for testing the forced degradation behavior of this drug in its combined dosage form, so testing the forced degradation behavior of this drug under various conditions such as hydrolytic (acidic, basic), oxidative, and thermal according to ICH Q1A (R2)[21] is an ongoing task. The key objectives of the research are to (1) build a way to indicate that is simple, particular, selective, sensitive, linear, accurate, robust, precise, and stable, and (2) perform a forced deterioration study in compliance with ICH guidelines. (3) To validate the established methods in compliance with ICH Q2 (aR1) guidelines.

EXPERIMENTAL

Materials and reagents

Sildenafil Citrate standard and Duloxetine HCl standard were obtained as a gift sample from Sunrise Pvt. Ltd. HPLC grade solvents Acetonitrile, Phosphate Buffer were purchased from Merck, Rankem.

Instrumentation

The HPLC was carried out on Shimadzu LC-10AT consisting of an auto-injector, sample manager and quaternary gradient pump with SPD20A PDA detector. The output signal was monitored and processed using LC-Solution software. A thermal degradation study was done using a hot air oven (Labline, India). Hydrolytic degradation was done using a digital water bath (Meta lab Scientific Industries Ltd., Mumbai, India). All pH measurements were carried out on a pH meter (Lab India, India), and weighing was done using a Mettler Toledo, Schwerzenbach, Switzerland. An ultrasonic bath sonicator (manufactured by, 5510, Branson Ultrasonic's Corporation, Danbury, CT, USA) was used for dissolving samples.

Conditions

The separation was accomplished on a Thermo scientific C_{18} BDS column (25 cm x 4.6 mm x 5 m) at pH 4 using Acetonitrile: Phosphate buffer (50:50% v/v) as the mobile phase. The mobile phase was filtered using 0.45 mm membrane filter paper & sonicated for 20 minutes.

Acetonitrile: Phosphate buffer (50:50% v/v) was used as a diluent. The flow rate was set to 1 mL/min and the detection wavelength (λ) at 290 nm. The run time was 7 minutes.

Preparation of Solutions

Preparation of standard stock solution of Sildenafil citrate

Sildenafilcitrate (25 mg) was weighed & transferred into a 25mLvolumetric flask (VF). 25mL of mobile phase was added and sonicated to dissolve. To obtain 100 µg/mL working standard solutions, a 1 mL aliquot of the stock solution was diluted to 10 mL with diluent.

Preparation of standard stock solution of Duloxetine HCl

Duloxetine HCl (25 mg) was weighed & transferred into a 25mLvolumetric flask (VF).15mL of mobile phase was added and sonicated to dissolve & make up the volume with mobile phase and mixed it. A 0.6 mL aliquot of the standard stock solution was diluted to 10 mL with diluent to make up the working standard solutions of 60 µg/mL.

Preparation of a standard stock solution of Sildenafil citrate & Duloxetine HCl

Sildenafil citrate (100 mg) and Duloxetine HCl (60 mg) were precisely weighed and transferred to a 100 mL volumetric flask, dissolved in the sufficient mobile phase, and diluted up to the mark with mobile phase to achieve a final concentration of 100 µg /mL Sildenafil citrate and 60 µg /mL Duloxetine HCl.

Preparation of the solution Sildenafil citrate & Duloxetine HCl

The sample was prepared by taking 20 tablets into mortale pestle, crushed it & the powder was taken into 100 ml of volumetric flask, diluted with 50 ml of mobile phase, placed in an ultrasonic water bath for 10 mins to achieve optimal solubility of the active components, and diluted to the mark (1000 µg /mL of SIL and 600 µg /mL of DULO). Soltion was filter with whatman filter paper no. 0.45 mm. From this, 1mL of this solution was taken into 10mL volumetric flask & the volume was made up to 10 mL with mobile phase. (SIL 100µg/mL and DULO 60µg/mL).

FORCED DEGRADATION STUDIES

Forced degradation experiments were carried out according to ICH Q1(A) R2 guidelines to determine the drug's stability under various stress conditions such as hydrolysis (acidic, basic, neutral), oxidation, & thermal.

Forced degradation sample stock preparation:

Sildenafil citrate (100 mg) and Duloxetine HCl (60 mg) were precisely weighed and transferred to a 100 mL volumetric flask, where they were dissolved in an appropriate amount of mobile phase (Acetonitrile: Phosphate buffer (50:50)) and diluted to the mark. (The solution

contains 1000 µg /mL Sildenafil citrate and 60 µg /mL Duloxetine HCl. The resulting solution was known as the standard degradation solution. Using 0.45mm membrane filter paper, the final solution was filtered.

Neutral hydrolysis

Neutral hydrolysis was performed using water. 1 mL of forced degradation sample stock preparation was taken and added into 10 mL of volumetric flask. This resulting solution was kept under waterbath at 70°C. After some time, cool the contents to room temperature before adding diluents to make up the volume. (SIL at 100 µg/mL and DULO at 60 µg/mL).

Acidic hydrolysis

Acidic hydrolysis was performed using 0.5N HCl. 1 mL of forced degradation sample stock preparation was taken and added into 10 mL of volumetric flask. Add 2 mL of 0.5N HCL solution and mix. This resulting solution was kept under waterbath at 70°C after 1 hour to cool at room temperature. Samples were neutralized with 2 mL of 0.5N NaOH & solution was diluted. (SIL 100 µg/mL and DULO 60 µg/mL).

Basic hydrolysis

Basic hydrolysis was performed using 0.5N NaOH. 1 mL of forced degradation sample stock preparation was taken and added into 10 mL of volumetric flask. Add 2 mL of 0.5N NaOH solution and mix. This resulting solution was kept under waterbath at 70°C after 1 hour to cool at room temperature. Samples were neutralized with 2 mL of 0.5N NaOH & solution was diluted. (SIL 100 µg/mL and DULO 60 µg/mL).

Peroxide degradation

Peroxide degradation was performed using 3% H2O2. Transfer 1mL offorced degradation sample stock preparation into 10mL of volumetric flask & add 2mL 3% H2O2 and put the volumetric flask in a water bath for 2 hours at 70°C, After the time period remove the flask from the waterbath and allow to cool at RT and then makeup the volume with diluent. (100µg/mL of SIL and 60µg/mL of DULO).

Thermal degradation

Thermal degradation was performed in a vacuum oven. Sildenafil citrate (100 mg) and Duloxetine HCl (60 mg) were precisely weighed, taken into petri plate & kept under vacuum oven at 105°C. and transferred to a 100 mL volumetric flask, where they were dissolved in an appropriate amount of mobile phase (Acetonitrile: Phosphate buffer (50:50)) and diluted to the mark. (The solution contains 1000 µg /mL Sildenafil citrate and 60 µg /mL Duloxetine HCl. The resulting solution was known as the standard degradation solution. Using 0.45mm membrane filter paper, the final solution was filtered.

METHOD VALIDATION

The following method was utilized & validated in accordance with ICH standards[ICH, 1996.][23]

System Suitability Parameters

Five times repeating injections (n=5) were used to establish system suitability in terms of retention time (tR), number of plates (NTP), tailing factor, and peak area using 100 µg /mL of SIL and 60µg /mL of DULO, which were produced from a stock solution of 1000 µg /mL of SIL and 600 µg /mL of DULO.

Linearity&Range (n=5)

The linearity response data was identified by examining five unique calibration curve levels ranging from 50 to 150µg /mL for Sildenafil citrate (50, 75, 100, 125, and 150µg /mL) and 30-90 µg /mL (30, 45, 60, 75, and 90µg /mL) for Duloxetine HCl. The calibration curve was plotted against peak area (AU) vs concentration (µg /mL).

Accuracy(n=3)

The recovery study was carried out using the standard addition technique, in which known amounts of standard powders of Sildenafil citrate& Duloxetine HCl were added to the pre-analyzed samples at 50%, 100%, and 150% levels.

Precision

The precision parameter was established using three variables: repeatability, intra-day precision, and inter-day precision.

Repeatability(n=6):

Method precision was established by analyzing six sample preparations under the same chromatographic condition as per method. Six replicates of sample were prepared at the test concentration and injected on the same equipment and on the same day. Relative standard deviation (RSD) or percentage coefficient of variation(%RSD) should not be more than 2%.

Intraday Precisionand Interday Precision

The intra day precision was performed by using 3 different concentrations 50, 100, and 150µg/mL for Sildenafil citrate (n=3) and 30, 60, and 90µg/mL for Duloxetine HCl (n=3) prepared from the stock solution of 100µg/mL of Sildenafil citrate and 60µg/mL of Duloxetine HCl & injected on the same day while in inter-day precision, the solution was injected on

three consecutive days.

LODand LOQ

The calibration curve approach was used to calculate the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the standard deviation of the response and the slope.

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where,
σ = Standard deviation of the intercept&
S = Slope of the calibrationcurve

Estimation of Sildenafil citrate & Duloxetine HCl in tablet dosage form by the proposed method (n=5)

Twenty tablets were weighed and crushed finely. 60 mg of Duloxetine and 100 mg of Sildenafil citrate are present in each tabletThe stock solution is made by dissolving equivalent to 10 mg of tablet powder in 10 ml mobile phase, placing it in an ultrasonic water bath for 10 minutes to ensure complete dissolution of the active components, and diluting it to the desired concentration with mobile phase (100µg/ml Sildenafil citrate and 60µg/ml Duloxetine HCl). Take 1 ml of this solution and add 1 ml of SIL from a standard stock solution of 100µg/ml land make up to 10 ml to get 100µg/ml Sildenafil citrate and 60µg/ml Duloxetine HCl were produced, applied to an HPLC column, and evaluated under optimum chromatographic conditions.

RESULT AND DISCUSSION

Optimization of the method

Many runs were performed using mobile phases consisted of solvents of varying polarity and concentration levels to establish a suitable mobile phase for the efficient separation of SIL & DULO. For the method development, various mobile phase systems, as well as concentration levels, were tested. The mobile phase consisting of Acetonitrile: phosphate buffer (50:50% v/v) with pH-4 provided the highest resolution with strong well-defined peaks and Rt values of 3.66 min± 0.02 and 5.28 min± 0.02 for SIL & DULO, respectively. The overlain chromatogram of the both drug were acquired on the HPLC instrument to determine the analytical wavelength for quantification. SIL and DULO demonstrated significant absorbance at around 290nm, chosen as the analytical wavelength for analysis.

Forced degradation studies

Optimization

In hydrolysis (acidic&basic), degradation was beginning with concentration (0.5N) & high temperature(T) (70°C) while peroxide degradation was beginning with low concentration(3%). The greatest concentration of NaOH was not used in basic hydrolysis since the sample was discovered to be unstable and easily precipitated out, thus a low concentration (0.5 N) was used. In thermal, degradation was beginning with a high temperature (105°C) because SIL and DULO melting point are 189-190°C and 118-122°C respectively. In acidic, basic, peroxide & thermal conditions, sample were analysed from 1 hrs to 24 hrs. In water & thermal conditions, less than 5 degradation was observed while in acidic, basic & peroxide conditions, degradation was performed. Detailed of force degradation were described in **Table 1.**

Table 1: Summary of Forced Degradation Studies of Sildenafil Citrate and Duloxetine Hydrochloride

Stress	*SIL*			*DULO*		
	Peak Area	*Retention Time*	*%Degradation*	*Peak Area*	*Retention Time*	*%Degradation*
As such	1402.21	3.65	-	883.87	5.280	-
Water hydrolysis	1340.20	3.653	4.42	873.16	5.277	1.21
Acid	1253.59	3.653	10.59	793.29	5.277	10.24
Base	1155.72	3.653	17.57	730.07	5.277	17.37
Oxidation	1180.84	3.650	15.78	749.02	5.270	
thermal	1398.20	3.657	0.28	843.57	5.283	4.55

Neutral hydrolysis

In neutral hydrolysis with water at 70°C for 3 hours, six degradation products were obtained: DP1 (degradation product), DP2, DP3, DP4, DP5, and DP6. DP1 and DP3 were possible degradation products with 4.42% and 1.21% degradation, respectively. (detailed shown in **Fig.1**).

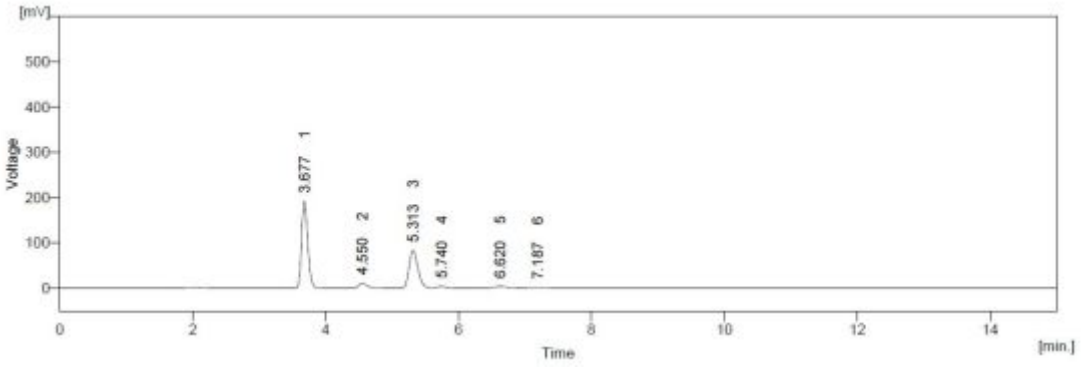

Fig .1 Chromatogram of Neural Hydrolysis of Standard mixture at 290 nm

Acidic hydrolysis

For 1 hour of acidic hydrolysis with 0.5 N HCl at 70°C, eight degradation products were obtained: DP1 (degradation product), DP2, DP3, DP4, DP5, DP6, DP7, and DP8. DP1 and

DP4 were possible degradation products, with 10.59% and 10.24% degradation, respectively. (detailed shown in **Fig.2**).

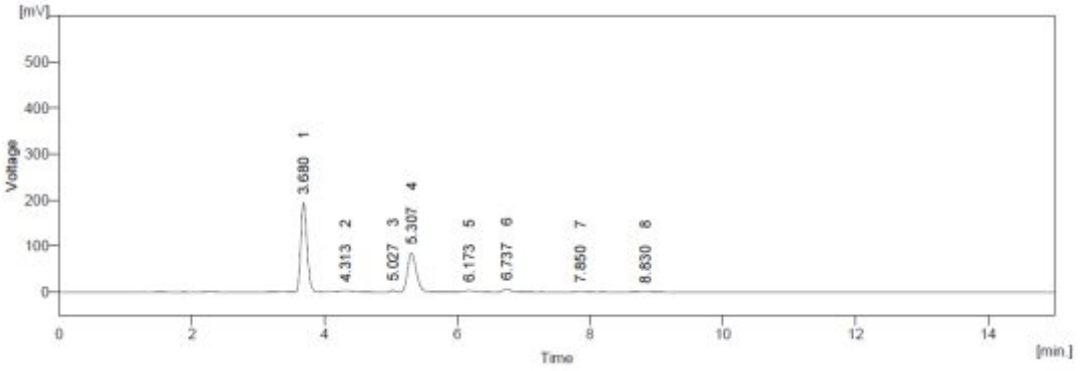

Fig .2 Chromatogram of Acid Degradation of Standard Mixture at 290 nm

Basic hydrolysis

Basic hydrolysis with 0.5 N NaOH at 70°C for 1 hour yielded eight degradation products: DP1 (degradation product), DP2, DP3, DP4, DP5, DP6, DP7, and DP8. DP1 and DP3 were possible degradation products with 17.57% and 17.37% degradation, respectively. (detailed shown in **Fig.3**).

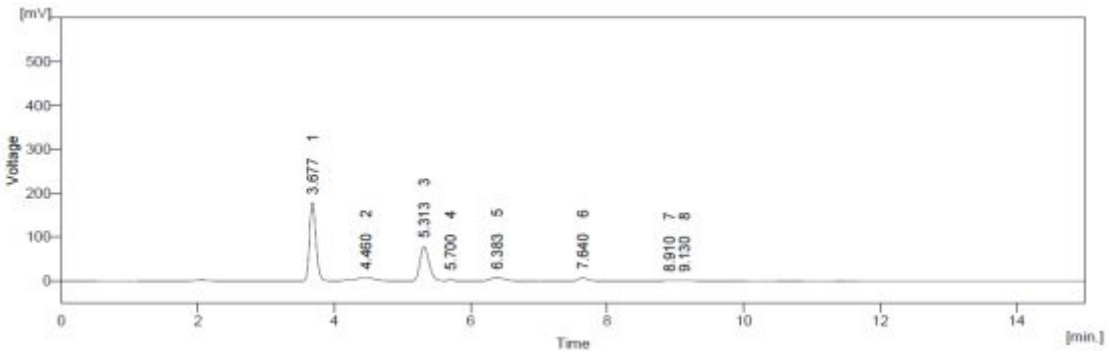

Fig .3 Chromatogram of Base Degradation of Standard Mixture at 290 nm

Peroxide degradation

Peroxide degradation using 3% H2O2 at room temperature for 2 hours yielded six degradation products: DP1 (degradation product), DP2, DP3, DP4, DP5, and DP6. DP1 and DP4 were possible degradation products with 15.78% and 15.25% degradation, respectively. (detailed shown in **Fig.4**).

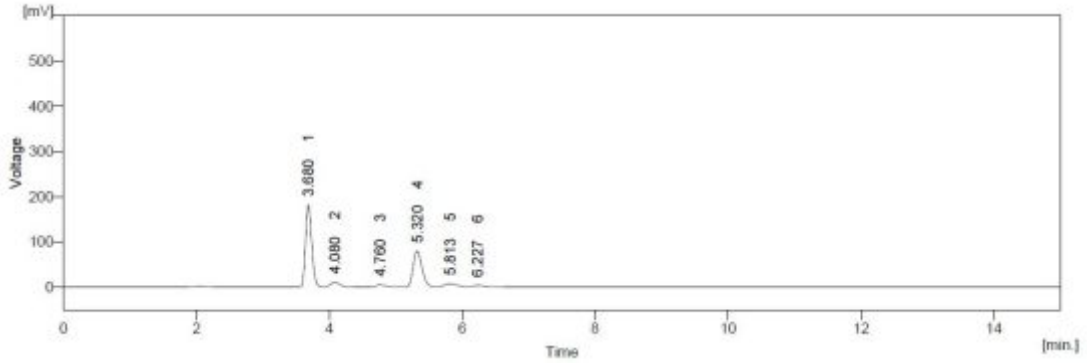

Fig.4 Chromatogram of Oxidation Degradation of Standard Mixture at 290nm

Thermal degradation

Thermal degradation at 105°C for 8 hours yielded six degradation products: DP1 (degradation product), DP2, DP3, DP4, DP5, and DP6. DP1 and DP5 were possible degradation products, with 0.28% and 4.55% degradation, respectively. (detailed shown in **Fig.5**).

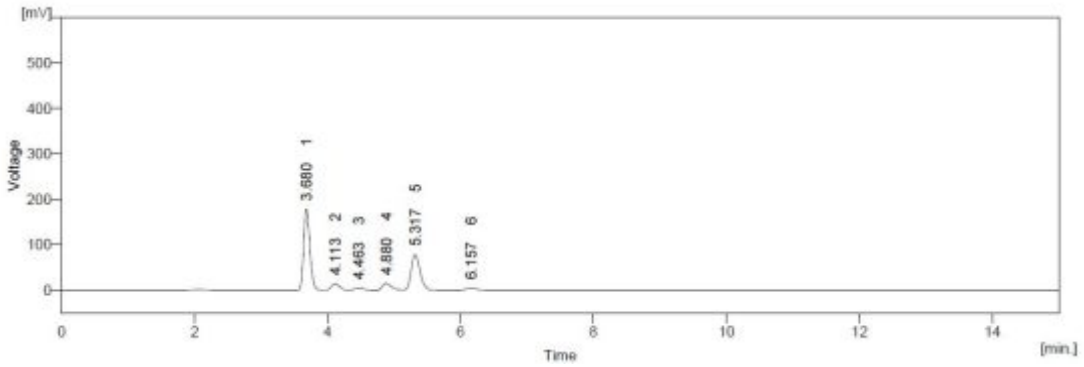

Fig.5 Chromatogram of Thermal Degradation of Standard Mixture at 290 nm

Method Validation

System Suitability Parameters

Table 2 shows the results of the system-suitability test parameters. The percentage RSD for all parameters was determined to be less than 2%. This implies that the system is suitable.

Table 2: System Suitability Parameters (SSP) for SIL & DULO (n=5)

System Suitability Parameters (SSP)	DRUGS	
	SIL±SD	DULO±SD
Retention time(min)	3.65±0.01	5.28±0.05
Tailing factor(T)	1.43±0.07	1.38±0.03
Number of theoretical plates (N)	7026±27.82	7280±35.32
Resolution (R)	7.65	

Linearity

The method was found linear in the range of 50-150µg/mL for SIL and in the range of 30-90µg/mL for DULO. **Table 3** displays the correlation coefficients, y-intercepts, and slopes of the regression lines for the 2 medicines. The linearity of the method wasproved by the value of the regression coefficient (R^2) of SIL and DULO as shown in **Fig 6 (a)** and **Fig 6 (b)** respectively.

Table 3: Linearity Data of SIL & DULO(n=5)

Parameters*	SIL	DULO
Linearity range	50-150 µg/mL	30-90 µg/mL
Linearity regression equation	y=40.187x+78.554	y=602.8x+681.35
Slope ± SD	14.107	14.844
Intercept ± SD	6.0688	2.7914
Correlation coefficient (r^2)	0.999	0.999

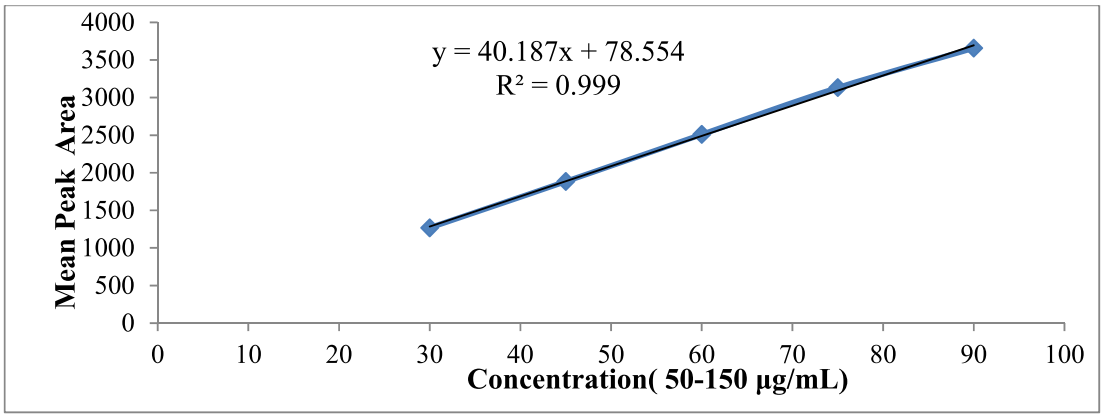

Fig. 6: (a)Calibration Curve for SIL (50-150 µg/mL)

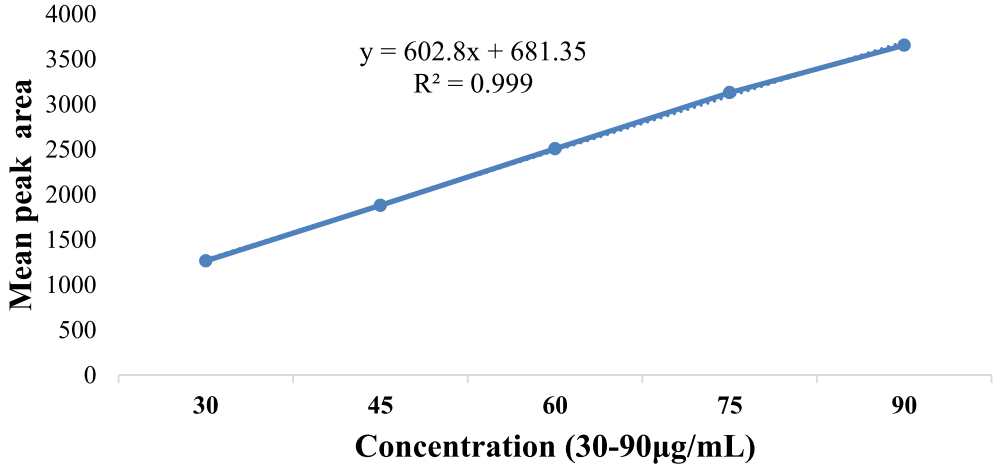

Fig. 6: (b) Calibration Curve for DULO (30-90µg/mL)

Specificity

The specificity of the proposed method was assessed by injecting about 20 µL of the SIL and DULO blank or working standard and sample solution into the HPLC system and recording chromatograms as shown in (**Fig.7 (a), (b), (c),** and **(d)**, respectively. under optimal chromatographic conditions. The chromatograms of standard SIL and DULO, as well as the sample, revealed two peaks with 100% peak area & Rtvalues of 3.66 min± 0.02 & 5.28 min± 0.02 for SIL and DULO, respectively.

Fig. 7 (a) Chromatogram of Blank

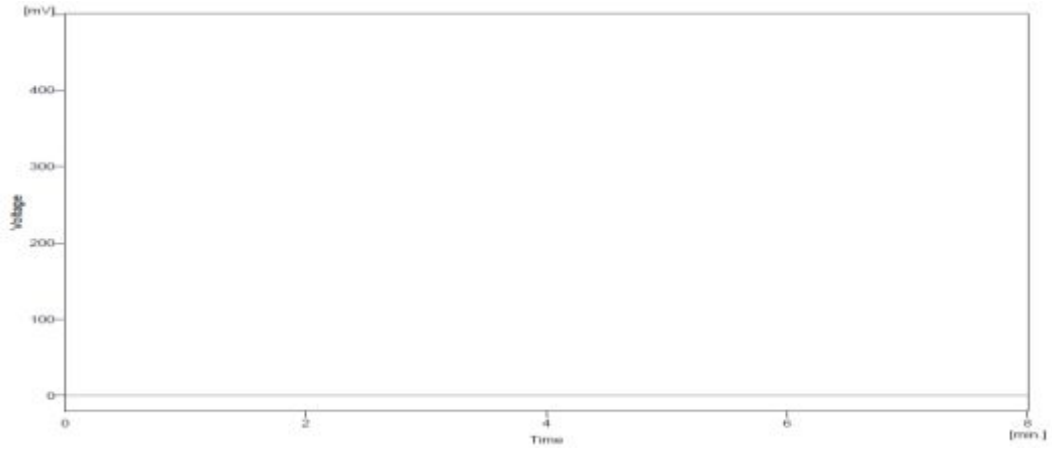

Fig. 7(b) Chromatogram of Standard SIL (100µg/mL)

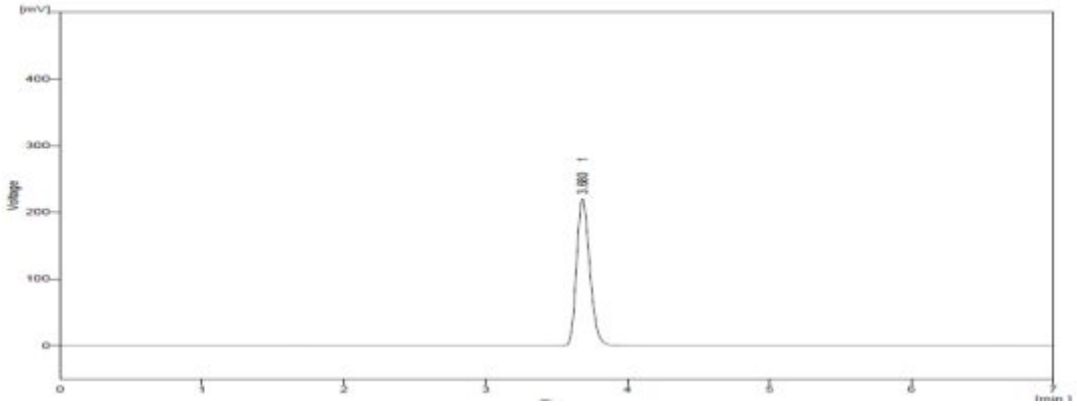

Fig. 7(c) Chromatogram of Standard DULO (60µg/mL)

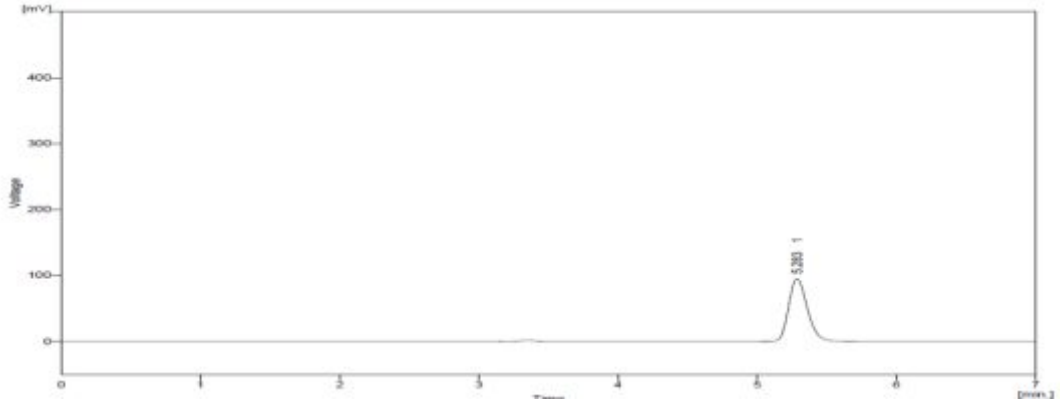

Fig. 7(d)Chromatograms of SIL (100 µg/mL) and DULO (60 µg/mL) Sample Solution

Accuracy

Tests for recovery were carried out at 50%, 100%, and 150% of the test concentration. At all three levels, the percentage recovery of SIL and DULO was considered to be satisfactory (**Table 4**). The percentage recoveries for SIL and DULO were discovered to be in the range of 100.22-100.57% and 100.35-100.72%, respectively.

Table 4: Accuracy Data of SIL & DULO (n=3)

Drug	Spike level%	Taken	Spiked Amount	Amount Recovered	SD	% recovery
SIL	50	50	75	75.31	0.52	100.39
	100	50	100	100.57	0.66	100.57
	150	50	125	125.26	0.80	100.22
DULO	50	30	45	45.26	0.52	100.56
	100	30	60	60.56	0.66	100.72
	150	30	75	75.25	0.80	100.35

Precision

Repeatability(n=6)

The repeatability data for SIL and DULO are shown in Table 9. %RSD for SIL and DULO were found to be 0.72% and 0.89% respectively. **Table 5** displays the repeatability outcomes.

Drug	Conc.	1	2	3	4	5	6	Standard Deviation	%Relative SD
SIL	**100**	1417.46	1430.46	1406.12	1416.75	1431.46	1410.45	10.32	0.72
DULO	**60**	892.46	896.41	883.21	890.23	894.45	907.47	8.00	0.89

Intraday precision and Inter day precision(n=3)

The data for intraday precision for SIL and DULOis shown in Table10. The % RSD for SIL and DULO was found to be0.98-1.74% and 0.99-1.74% respectively. The % RSD was found to be 0.64-1.06% and 0.64-1.07% for SIL and DULO respectively. The results of repeatability are as shown in

Table 6: Intraday & Interday Precision of SIL & DULO (n=3)

Drug		SIL			DULO	
Conc. (µg/ml)	**50**	**100**	**150**	**30**	**60**	**90**
Intraday precision						
Mean peak area	706.1757	1411.23	2121.864	444.9153	889.624	1340.416
± S.D.	7.54	12.01	13.72	4.76	7.57	8.63
% R.S.D	1.06	0.85	0.64	1.07	0.88	0.64
Interday precision						
Mean peak area	704.815	1416.938	2113.992	444.0517	893.2373	1333.567
± S.D.	6.96	14.59	36.89	4.40	9.20	23.26
% R.S.D	0.98	1.03	1.74	0.99	1.03	1.74

The % RSD for repeatability, intra-day precision, and inter-day precision were all less than two, indicating that the approach was precise.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD & LOQ were found to be 0.540 & 1.639 µg/mL for SIL and 6.230 & 18.879 µg/mL for DULO respectively. Similarly, LOQ for SIL and DULO were found to be 1.639 and 18.879 respectively, indicating the sensitivity of the developed method.

ANALYSIS OF MARKETED FORMULATION

The new approach was used to examine the marketed formulations that are SIL, and DULO. The sample chromatogram exhibited just 2 peaks at Rt values of 3.66 min & 5.28 min for SIL and DULO, implying that no excipients included in the tablet formulation interfered. The SIL and DULO concentrations were determined by comparing the samples peak areas to those of the standard. (**Table 7**).

Table 7: Assay Data of SIL & DULO (n=5)

Drug name	**Amt added per 1 ml of solution**	**% Amt found**	**SD**	**RSD**
SIL	100 mg	100.54%	1.29	1.29
DULO	60 mg	100.40 %	1.14	1.14

CONCLUSION

The simple, specific, selective, linear, accurate, robust, sensitive & stability indicating method was developed. The forced degradation study was performed as per ICH Q1A (R2) guideline. In acidic conditions, satisfactory deterioration was attained(10.59% for SIL & 10.24% for DULO), DP1& DP4 werefoundas a potentdegradation product, Inbasiccondition(17.57% for SIL & 17.37% for DULO), DP1& DP3 were found as apotentdegradationproduct, Inoxidation(15.78% for SIL & 15.25% for DULO), DP1& DP4 were found as a potent degradation product,Inwater hydrolysis(4.42% for SIL &1.21% for DULO), DP1& DP3 were found as a potent degradation product,The degradation was achieved in thermal condition(4.55% for DULO), DP5 wasb found s a potent degradation product. There was no degradation found in the thermal condition for Sildenafil Citrate.

LIST OF ABBREVIATIONS

I. SIL,Sildenafil

II. DULO, Duloxetine

REFRENCES

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