Development and validation of RP-HPLC method for simultaneous estimation of Amitriptyline Hydrochloride and Propranolol Hydrochloride in pharmaceutical dosage form.

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ABSTRACT:

Reverse phase high performance liquid chromatography method has been developed and validated for simultaneous estimation of Amitriptyline Hydrochloride and Propranolol Hydrochloride in dosage form. This method uses C18 agilent column with 4.6 x 250mm length and 5µm particle size of packing material. Mobile phase is methanol: water pH 6 with TEA (70:30) with 1ml/min flow rate and 20µl volume injected. UV detection was carried out at 216 nm and the column temperature is 250C. The retention time of Amitriptyline Hydrochloride was 3.882 min. and 6.384 min of Propranolol Hydrochloride. The method is validated and calibration curve observed was linear in the concentration range of 5-25µg/ml for Amitriptyline Hydrochloride and 20-100µg/ml for Propranolol Hydrochloride. The method is validated for linearity, accuracy, precision, limit of detection and quantification, ruggedness and robustness.

Key Words: Amitriptyline Hydrochloride, Propranolol Hydrochloride, RP-HPLC, UV Detection.

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1. INTRODUCTION

Amitriptyline Hydrochloride is tricyclic antidepressant drug and is approved for the treatment of major depression.[1] It is 3-(10,11-dihydro-5Hdibenzo[a,d]cyclohept-5-ylidene) propyl dimethylamine hydrochloride. It increase noradrenergic or serotonergic neurotransmission by blocking norepinephrine or serotonin transporter at presynaptic terminals.[2] It contain tricyclic ring system with an alkyl amine substituent on the central ring. may cause sedation.[3] Propranolol Hydrochloride is a non-selective β-adrenergic antagonist with no intrinsic sympathomimetic activity. It is used in the hypertension, angina pectoris, myocardial infraction and cardiac arrhythmias.[4] It is highly lipophilic and absorbed after oral administration.[5] Propranolol Hydrochloride (RS)-1-[(1- methyl ethyl) amino]-3-(napthalen-1-yloxy) propan-2-hydrochloride is antihypertensive. It competitively blocks both B1

&B2 adrenergic receptors. [6] No method was reported for simultaneous estimation of Amitriptyline Hydrochloride and Propranolol Hydrochloride combination. Therefore in this research work we have reported RP-HPLC method for simultaneous estimation of Amitriptyline Hydrochloride and Propranolol Hydrochloride is developed and validated as per ICH guidelines. This method is simple,precise and accurate. High Performance Liquid Chromatography is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. Liquid chromatography is separation technique based on the difference in a distribution of the component between two non-miscible phases in which liquid mobile phase elute through a stationary phase in column. The HPLC is based on mechanism of partition, adsorption and ion exchange [7].

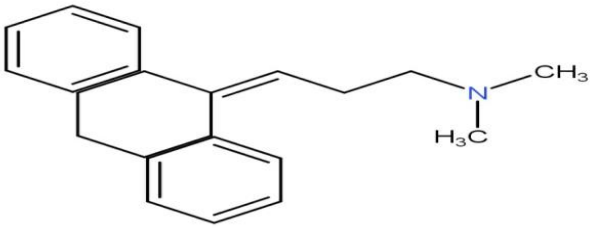

Fig.1 Chemical Structure of Amitriptyline

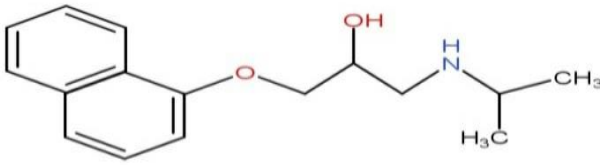

Fig.2 Chemical Structure of Propranolol

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Working standard of Amitriptyline was obtained from Dhamtec Pharma and Consultants and Propranolol was obtained from Swaroop Research Private Limited. Fixed dose of Amitriptyline (10 mg) + Propranolol (40 mg) containing tablet AmNurite Beta was purchased from Tata 1 mg online platform. All HPLC grade chemicals purchased from Merck Chemicals, India. Water used was double distilled and filtered through a 0.45 µ filter disc. HPLC system consisted of Agilent Technology 1200 series quaternary gradient with Agilent SPD-M20A prominence diode array detector and a 515 auto injector. Data were acquired and processed by use of EZ Chrome Elite software. The chromatographic separations were carried out on a C18 Column (250×4.6mm, 5 µm).

2.2 Preparation of Standard Stock Solution:

A 5 mg amount Amitriptyline and 20 mg Propranolol of reference substance was accurately weighed and dissolved in 10 ml methanol in volumetric flask to obtain 500 µg/ml Amitriptyline 2000 µg/ml Propranolol concentration of stock solution. From stock solution by the serial dilution we prepared required concentrations of 5, 10, 15, 20, and 25 ppm for Amitriptyline and 20, 40, 60, 80 and 100 ppm for Propranolol.

2.3 Preparation of sample solution:

Twenty tablets containing 10 mg Amitriptyline Hydrochloride and 40 mg Propranolol Hydrochloride was weighed and average weight was calculated. Tablets were finely crushed and equivalent weight (137.5 mg) to each tablet was taken into 100 ml volumetric flask. 50 ml of methanol was added and solution was sonicated for 30 minutes. Volume was made up to the mark. After filtration, 0.1 ml solution pipetted out and volume made up to 10 ml with mobile phase, this gives concentration of 5 ppm of Amitriptyline Hydrochloride and 20 ppm of Propranolol Hydrochloride.

2.4 Preparation of mobile phase

For the preparation of the mobile phase, 70 ml of methanol and 30 ml of water are mixed and pH 6 adjusted with TEA and this mobile phase filtered through 0.45-micron filter paper.

2.5 Chromatographic Conditions

The mobile phase used Methanol: Water (70:30 v/v) of pH 6.0 was maintained by TEA. Injections were carried out using a 20 µl loop at ambient temperature and the flow rate was 1 ml/min. Detection was performed at 216 nm with 15.0 min runtime.

2.6 Method Validation [8]:

Method validation was performed according to ICH specifications, range of linearity, precision, robustness, Recovery, LOD and LOQ and Tablet Assay.

2.6.1 Specificity: For specificity standard drug and sample solutions were analyzed. The retention time of drugs present in the sample solution was compared with a standard solution.

2.6.2 Linearity: In linearity studies, 5 different concentrations of Amitriptyline and Propranolol

Were injected. Concentrations in the range of 5, 10, 15, 20, 25 µg/ml of Amitriptyline and 20, 40,60, 80, 100 µg/ml of Propranolol. The calibration curve was constructed by plotting the peak area against the concentration of drugs.

2.6.3 Precision: Precision is the closeness of measurements of the same item to each other. precision studies were done by intraday and interday variations. For intraday precision, one concentration (10+40 µg/mL) was injected (each injection 6 times) in the same equipment on the

same day. Inter-day precision was carried out on different days. From obtained data of peak areas, the mean was determined, and then the standard deviation and relative standard deviation.

2.6.4 Limit of detection (LOD) and Limit of quantization (LOQ): LOD is the smallest concentration of analyte that gives a response that is measurable. LOQ is the smallest concentration of analyte that gives a response that can be quantified. This was established at a signal-to-noise ratio. LOD and LOQ were determined by progressively injecting the smallest concentration of a standard solution.

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

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

Where, σ= Standard deviation of y- intercept of calibration line.

S = slope of the calibration curve.

2.6.5 Accuracy: The accuracy of an analytical procedure expresses the closeness of the agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Closeness of the measured value to the true value is the accuracy of the method for the sample. By recovery studies accuracy of the method was determined. This was carried out at three different concentrations levels 80%, 100%, and 120%.

Accuracy solution preparation: Accuracy was carried out at three different concentration levels, which are 80%, 100%, and 120%. Take 5+20 µg/ml solution.

2.6.6 Assay: For assay, solution was prepared by weighing 20 tablets that are finely crushed and their weight was 5.5 mg. The average powder weight of 20 tablets was calculated which is 0.275 mg. An equivalent weight of 20 mg (137.5 mg) is taken and dissolved in 10 ml of methanol. After filtration, 0.2 ml solution pipette out and volume made up to 10 ml with mobile phase, which gives the concentration of 10 ppm of Amitriptyline and 40 ppm of Propranolol. This stock 2 solution was injected for assay.

2.6.7 Ruggedness: To study the ruggedness parameter same concentration of the solution was injected by two different operators on HPLC. For ruggedness concentration of 10+40 µg/ml was injected by different operators.

2.6.8 Robustness: For robustness, some parameters like mobile phase concentration, flow rate, and wavelength were deliberately changed and data were recorded. It was determined by making small changes in chromatographic conditions. For robustness concentration of 10+40 µg/ml was injected at different flow rate, wavelength and different mobile phase composition.

3. RESULTS AND DISCUSSION

System Suitability

Optimization of efficiency for chromatographic separation the quality of the chromatography was monitored by applying the system suitability tests like capacity factor, tailing factor and theoretical plates. The system suitability acceptance criteria set for each validation run were: capacity factor >2.0, tailing factor ≤2.0 and theoretical plates >2000. For the analytic peak area the relative standard deviation (R.S.D) of two consecutive injections was < 2.0%. For reference substance solution chromatogram obtained is presented. System suitability parameters were shown in Table1.

Table.1- System suitability parameter

Mobile Phase	Methanol: Water pH 6 with TEA (70:30)
Pump mode	Gradient
PH	6.0
Diluents	Methanol
Column	C_{18} Column (250×4.6 mm, 5µm)
Column temperature	Ambient
Wavelength	216 nm
Injection volume	20µl
Flow rate	1 ml/min
Run time	15 minutes
Retention time	Amitriptyline- 3.882 min Propranolol- 6.384 min

3.1 Specificity

For specificity standard drug and sample solutions were analyzed. Retention time of drugs present in sample solution was compared with standard solution. Chromatogram of tablet observed 3.882 at and 6.384 min. which shows no interference of excipients present in tablet.

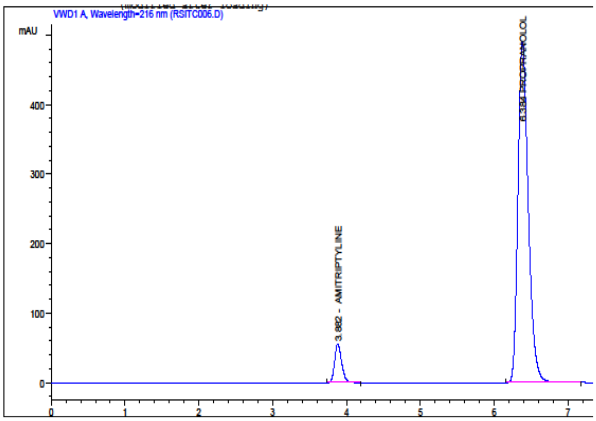

Fig1. Chromatogram of Amitriptyline and Propranolol standard

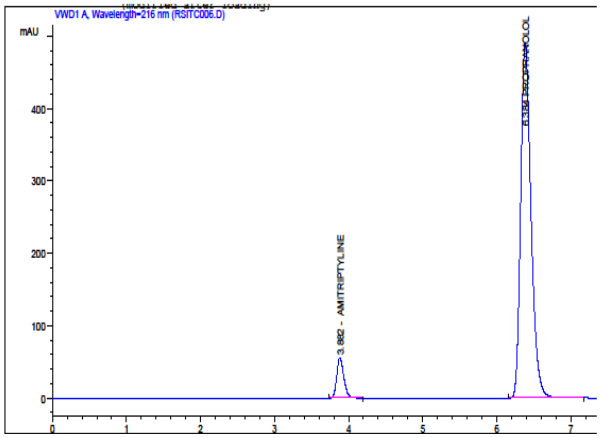

Fig.2. Chromatogram of Amitriptyline and Propranolol sample

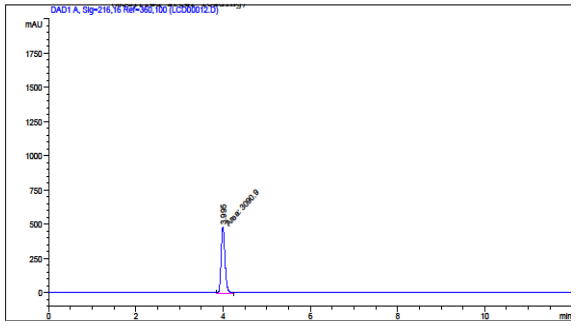

Fig.3. Chromatogram of Amitriptyline

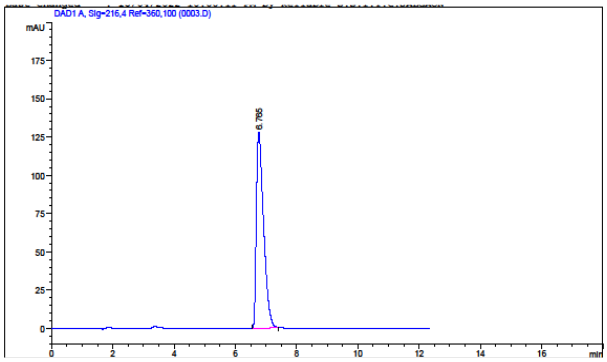

Fig.4. Chromatogram of Propranolol

3.2 Range of linearity

Amitriptyline and Propranolol standard curves were constructed using five different standard concentrations in a range of 5, 10, 15, 20, 25µg/ml and 20, 40, 60, 80, 100µg/ml respectively. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equation of Amitriptyline; y = 22.207x+12.083 (R^2 = 0.995) and Propranolol; y = 72.027x+197.81 (R^2 = 0.998). Linearity values are shown in Table 2 and 3

Table.2- Range of linearity of Amitriptyline

Concentration of Amitriptyline in ppm	Peak area
5	124.6
10	234.35
15	342.22
20	455.64
25	569.13

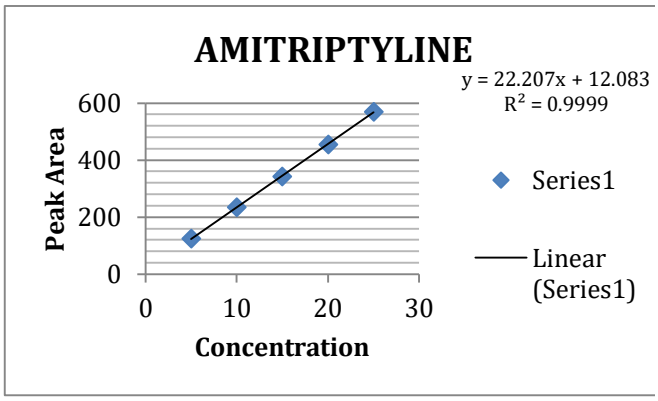

Fig. 4 – Linearity of Amitriptyline

Table 3 – Range of Linearity of Propranolol

Concentration of Propranolol in ppm	Peak area
20	1657.35
40	3086.71
60	4466.61
80	5966.1
100	7420.33

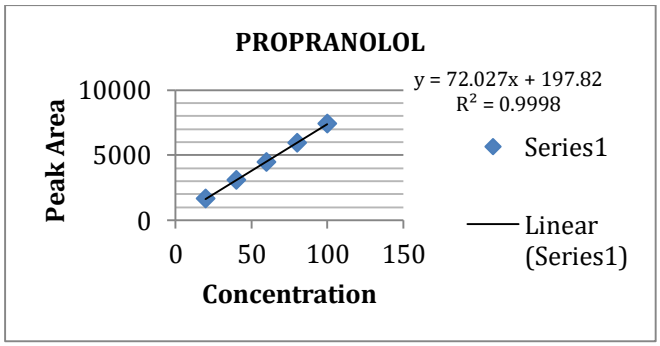

Fig. 5. – Linearity of Propranolol

3.3 Precision

Precision is the closeness of measurements of the same item to each other. Precision studies were done by intraday and interday variations. For intraday precision, one concentration (10+40 g/mL) was injected (each injection 6 times) in the same equipment on the same day. Inter-day precision was carried out on different days. From obtained data of peak areas, the mean was determined, and then the standard deviation and relative standard deviation.

Table 4. Result of Amitriptyline intraday and interday precision

Sample	Injection	Intraday Precision	Interday Precision
		Peak Area	
Amitriptyline	1	236.9942	236.9842
	2	236.9950	236.9850
	3	236.7780	236.7980
	4	236.6600	236.8000
	5	236.482	236.4820
	6	236.9000	236.9010
	Mean	236.7915	236.8250
	Standard Deviation	0.2130	0.1874
	Relative Standard Deviation	0.09%	0.08%

Table No. 5 Result of Propranolol intraday and interday precision

Sample	Injection	Intraday Precision	Interday Precision
		Peak Area	
Propranolol	1	3080.8862	3080.8762
	2	3080.8450	3080.8490
	3	3080.8500	3080.8300
	4	3080.5670	3080.5670
	5	3080.8100	3080.8000
	6	3080.2300	3080.2400
	Mean	3080.6980	3080.6937
	Standard Deviation	0.2564	0.2486
	Relative Standard Deviation	0.01%	0.01%

3.4 Limit of Detection and Limit of Quantification

LOD is the smallest concentration of analyte that gives response which is measurable. LOQ is the smallest concentrations of analyte that gives response which can be quantify. This was established at signal to noise ratio. LOD and LOQ were determined by progressively injecting smallest concentration of standard solution.

Table No. 7. Results of LOD and LOQ

Drugs	LOD (µg/ml)	LOQ (µg/ml)
Amitriptyline	0.33	1.00
Propranolol	1.59	4.83

3.5 Accuracy

By recovery studies the accuracy of method was determined. This was carried out at three different concentrations levels at 80%, 100% and 120%. Results of accuracy are given in following table.

Table No. 8. Accuracy results

Drug	Peak Area	Spike Level	Conc. Added (µg/ml)	Conc. Found (µg/ml)	% Recovery	Mean % Recovery	Standard Deviation	Relative Standard Deviation
Amitriptyline	211.436	80	4	3.97	99.42	99.34	0.2731	0.275%
	211.578			3.98	99.57			
	211.738			3.96	99.04			
	232.738	100	5	4.93	98.72	98.53	0.1960	0.199%
	232.564			4.92	98.56			
	232.100			4.91	98.33			
	256.273	120	6	5.99	99.93	99.93	0.1101	0.11%
	256.430			6.00	100.05			
	256.150			5.99	99.83			
Propranolol	2791.130	80	16	16.00	100	99.74	0.4827	0.48%
	2791.375			16.00	100.05			
	2781.450			15.87	99.19			
	3098.828	100	20	20.28	101.40	100.90	0.8372	0.83%
	3098.541			20.27	101.38			
	3378.100			19.98	99.94			
	3357.781	120	24	23.87	99.46	99.9	0.7794	0.78%
	3357.465			23.86	99.44			
	3380.900			24.19	100.80			

3.6 Assay

Assay results of Amitriptyline and Propranolol in tablet dosage form are given in following table. Results indicate suitability of method for routine analysis of Amitriptyline and Propranolol from combined dosage form.

Table No.9. Assay results

Drug	Label Claim	Amount Estimated	% Amount Estimated
Amitriptyline (10 µg/ml)	10 mg	10.01 mg	100.1%
Propranolol (40 µg/ml)	40 mg	40.10 mg	100.27%

3.7 Ruggedness

To study ruggedness parameter same concentration of solution was injected by two different operators on different HPLC instruments like Agilent HPLC and Shimadzu HPLC. Obtained results for this method are mentioned in following table.

Table No. 10. Ruggedness result

Analyst	Drugs	Retention Time	Theoretical Plates	Tailing Factor	Resolution
Analyst 1	Amitriptyline (10 ppm)	3.85	8752	0.76	-
	Propranolol (40 ppm)	6.34	10866	0.71	12.14
Analyst 2	Amitriptyline (10 ppm)	3.858	9446	0.76	-
	Propranolol (40 ppm)	6.340	12059	0.71	12.72

3.8 Robustness

For robustness some parameters like mobile phase concentration, flow rate, wavelength were deliberately changed and data were recorded. It was determined by making small changes in chromatographic conditions. Results of robustness are given in table no. 11.

Table No. 11. Result of robustness with changes in flow rate

Flow Rate	Drug	Theoretical Concentration (ppm)	Peak Area	Peak Area Mean	Retention Time	% Relative Standard Deviation
0.9 ml/min	Amitriptyline	10	234.355	234.365	3.897	0.01
			234.400			
			234.340			
	Propranolol	40	3086.7168	3086.388	6.437	0.02
			3086.7100			
			3086.7400			
1.1 ml/min	Amitriptyline	10	234.465	234.431	3.870	0.14
			234.450			
			234.380			
	Propranolol	40	3086.6900	3086.381	6.428	0.01
			3086.7250			
			3085.7300			

Table No. 12. Result of robustness with changes in wavelength

Wavelength	Drug	Theoretical Concentration (ppm)	Peak Area	Peak Area Mean	Retention Time	% Relative Standard Deviation
215 nm	Amitriptyline	10	234.250	234.0	3.797	0.25
			234.430			
			233.320			
	Propranolol	40	3085.9800	3086.45	6.115	0.01
			3086.7100			
			3086.7350			
217nm	Amitriptyline	10	233.560	234.146	3.898	0.26
			234.100			
			234.780			
	Propranolol	40	3085.9650	3086.5083	6.127	0.02
			3086.7300			
			3086.8300			

Table No. 13 Result of robustness with changes in mobile phase composition

Mobile Phase Composition	Drug	Theoretical Concentration (ppm)	Peak Area	Peak Area Mean	Retention Time	% Relative Standard Deviation
Methanol: water (69:31)	Amitriptyline	10	234.360	234.11	3.842	0.27
			234.570			
			234.400			
	Propranolol	40	3085.8750	3086.502	6.190	0.02
			3086.7940			
			3086.8370			
Methanol: water (71:29)	Amitriptyline	10	234.360	234.02	4.016	0.31
			234.520			
			234.180			
	Propranolol	40	3084.7690	3086.765	6.899	0.03
			3085.7805			
			3086.7480			

4. Conclusion

The present study shows that developed RP-HPLC method for the determination of Amitriptyline and Propranolol from pharmaceutical dosage form is accurate, robust, simple and precise. As obtained results are within accepted limit. This method is versatile as results of standard deviation, relative standard deviation and regression values are within acceptable limit. Developed method was validated as per ICH guidelines and it could be convenient for routine quality control analysis of Amitriptyline and Propranolol from their tablet dosage form.

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