NOVEL RP-HPLC METHOD FOR THE QUANTIFICATION DETERMINATION OF CLONAZEPAM AND PROPRANOLOL IN BULK FORM AND MARKETED FORMULATION

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ABSTRACT: *A new analytical simple, rapid, precise, accurate and sensitive reverse phase liquid chromatographic method has been developed for the simultaneous determination of Clonazepam and Propranolol in bulk and pharmaceutical dosage form dosage form. The chromatographic method was standardized using Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. i.d. column with UV detection at 255nm and Methanol: Phosphate buffer (0.02M) with 55:45 (pH-2.6) ratios at a flow rate of 1.0 ml/ min. Different analytical performance parameters such as precision, accuracy, limit of detection, limit of quantification and robustness were determined according to International Conference on Harmonization (ICH) guidelines. The method was linear over the range of 0-14µg/ml for Clonazepam and 0-28µg/ml for Propranolol. The recovery was in the range of 98% to 102%. The LOD was found to be 0.06 µg/ml and 0.09 µg/ml for Clonazepam and Propranolol respectively. The LOQ was found to be 0.18 µg/ml and 0.27 µg/ml for Clonazepam and Propranolol. The proposed method was successfully applied to the simultaneous determination of Clonazepam and Propranolol in bulk and pharmaceutical dosage form.*
Keywords: *RP-HPLC, Clonazepam and Propranolol, ICH Guidelines, Accuracy, Precision.*

I. INTRODUCTION

Clonazepam, sold under the brand Klonopin among others, is a medication used to prevent and treat seizures, panic disorder, anxiety, and the movement disorder known as akathisia. It is a tranquilizer of the benzodiazepine class. It is taken by mouth. Effects begin within one hour and last between six and twelve hours. Clonazepam[1] is a DEA Schedule IV controlled substance. Substances in the DEA Schedule IV have a low potential for abuse relative to substances in Schedule III. Clonazepam is a benzodiazepine used predominantly as an anticonvulsant as adjunctive therapy in management of epilepsy. Therapy with clonazepam[2] is not associated with serum aminotransferase elevations, and clinically apparent liver injury from clonazepam, if it occurs at all, must be exceedingly rare. Clonazepam is a synthetic benzodiazepine derivative used for myotonic or atonic seizures, absence seizures, and photosensitive epilepsy, anticonvulsant Clonazepam[3] appears to enhance gamma-aminobutyric acid receptor responses, although its mechanism of action is not clearly understood. It is seldom effective in generalized tonic-clonic or partial seizures. A benzodiazepine used to treat various seizures, including myotonic or atonic seizures, photosensitive epilepsy, and absence seizures, although tolerance may develop. The agent has also been indicated for treating panic disorder. The mechanism of action appears to involve the enhancement of gamma-aminobutyric acid receptor responses. The IUPAC Name of Clonazepam is 5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one. The Chemical Structure of Clonazepam is as follows

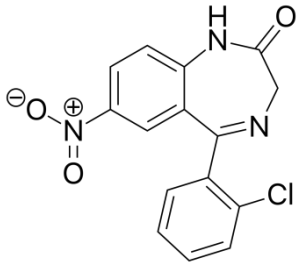

Fig.1. Chemical Structure of Clonazepam

Propranolol is a nonselective beta-adrenergic receptor blocker (beta-blocker) that is widely used for the therapy of hypertension, cardiac arrhythmias, angina pectoris and hyperthyroidism. Propranolol has yet to be convincingly associated with clinically apparent liver injury and is often used in patients with liver disease and cirrhosis. Propranolol[4] is a propanolamine that is propan-2-ol substituted by a propan-2-ylamino group at position 1 and a naphthalen-1-yloxy group at position 3. It has a role as a beta-adrenergic antagonist, an anxiolytic drug, an anti-

arrhythmia drug, a vasodilator agent, an antihypertensive agent, a xenobiotic, an environmental contaminant and a human blood serum metabolite. It is a secondary amine, a propanolamine and a member of naphthalenes. It derives from a 1-naphthol. Propranolol[5] is a racemic mixture of 2 enantiomers where the S (-)-enantiomer has approximately 100 times the binding affinity for beta adrenergic receptors. Propranolol[6] is used to treat a number of conditions but most commonly is used for hypertension. Propranolol was granted FDA approval on 13 November 1967. The IUPAC Name of Propranolol is 1-naphthalen-1-yloxy-3-(propan-2-ylamino) propan-2-ol. The Chemical Structure of Propranolol is as following

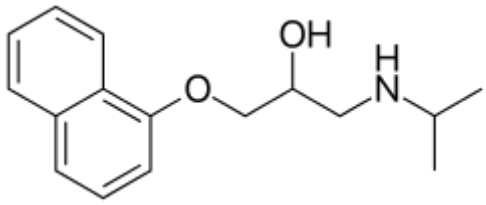

Fig. 2. Chemical Structure of Propranolol

Several HPLC methods[19-21] using a variety of columns and detection techniques have been reported on Clonazepam and Propranolol in combination with other drugs and physical and chemical stability studies. In the present study, novel method was developed with suitable mobile phase as solvent in HPLC; it is a simple method to study, detect and separate the Clonazepam and Propranolol from mixture of compounds and can be adopted for regular quality assessment in pharmaceutical industry and scientific laboratories.

II. EXPERIMENTAL

Materials and Method

The unique separation can be achieved with the HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters), T60-LAB INDIA UV – Vis spectrophotometer, Electronic Balance (SHIMADZU ATY224), Ultra Sonicator (Wensar wuc-2L), Thermal Oven, Symmetry ODS RP C18,5µm, 15mm x 4.6mm i.d., PH Analyzer (ELICO) and Vacuum filtration kit (BOROSIL).

Chemicals and Reagents

The chemical and reagents are used for the method development and validation of Clonazepam and Propranolol are Doubled distilled water, HPLC Grade Water, Methanol, Hydrochloric Acid, Acetonitrile and Sodium Hydroxide etc.

Method Development

Selection of Wavelength

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 is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Clonazepam and Propranolol, so that the same wave number can be utilized in HPLC UV detector for estimating the Clonazepam and Propranolol. The scanned UV spectrum is attached in the following page.

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Clonazepam standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

25 mg of Propranolol standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Preparation of Phosphate buffer (PH: 2.6):

Weighed 0.50 grams of di-sodium hydrogen phosphate and 0.301 grams of potassium dihydrogen phosphate was taken into a 1000 ml beaker, dissolved and diluted to 1000 ml with HPLC water, adjusted the pH to 2.6 with orthophosphoric acid.

Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Methanol and Phosphate buffer (0.02M, pH-2.6) at the volume of 1000ml. 550ml of Methanol and 450ml of Phosphate buffer were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Preparation of Standard Solutions:

10 mg of Clonazepam and Propranolol was weighed accurately and transferred into 10 ml volumetric flask. About 10 ml mobile phase was added and sonicated to dissolve. The volume was made up to the mark with same solvent.

The final solution contained about 10µg/ml and 10µg/ml of Clonazepam and Propranolol respectively.

Conclusion: The selected and optimized mobile phase was Methanol: Phosphate buffer (0.02M, pH-2.6) with 55:45 ratio and conditions optimized were flow rate (1.0 ml/minute), wavelength (255nm), Run time was 07 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Method Validation

1. System Suitability:

10 mg of Clonazepam and 10 mg of Propranolol methane working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and add about 2 ml of diluent and sonicated to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further 1 ml of Clonazepam and Propranolol methane was pipetted out from the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

2. Linearity and Range

Method:

Preparation of stock solution:

Accurately 10 mg of Clonazepam and 10 mg of Propranolol API Drug were weighed & sample were transferred into a 10ml clean dry volumetric flask and about 9ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (6ppm of Clonazepam & 12pm of Propranolol):

1ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – II (8ppm of Clonazepam & 16ppm of Propranolol):

2ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – III (10ppm of Clonazepam & 20ppm of Propranolol):

3ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – IV (12ppm of Clonazepam & 24ppm of Propranolol):

4ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – V (14ppm of Clonazepam & 28ppm of Propranolol)

5ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

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.

3. Accuracy:

Preparation of standard solution (Clonazepam and Propranolol):

Accurately weighed 10mg of Clonazepam and 10mg of Propranolol working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks. About 7mL and 10ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3ml and 0.3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluents.

Preparation of Sample solutions:

For preparation of 80% solution (With respect to target Assay concentration):

Accurately 8mg of Clonazepam and 8mg of Propranolol working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. Further 3ml and 0.3ml of the above Clonazepam and Propranolol stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately 10mg of Clonazepam and 10mg of Propranolol working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. Further 3ml and 0.3ml of the above Clonazepam and Propranolol stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

For preparation of 120% solution (With respect to target Assay concentration):

Accurately 12mg of Clonazepam and 12mg of Propranolol working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated

to dissolve it completely and made volume up to the mark with the same solvent. Further 3ml and 0.3ml of the above Clonazepam and Propranolol stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure:

The standard solution, Accuracy -80%, Accuracy -100% and Accuracy -120% solutions were injected. The Amount found and Amount added for Clonazepam & Propranolol and the individual recovery and mean recovery values were calculated.

4. Precision:

i) Repeatability

Preparation of standard stock solution:

Accurately 10 mg of Clonazepam and 10mg of Propranolol working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further it was pipette (3ml and 0.3ml) into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

The standard solution was injected for five times and the areas for all five injections in HPLC were measured. The %RSD for the area of six replicate injections was found to be within the specified limits.

ii) Intermediate precision

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

Preparation of standard stock solution:

Accurately 10 mg of Clonazepam and 10mg of Propranolol working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further this Stock was pipette (3ml and 0.3ml) into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for six times and the area for all six injections measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

5. Method Robustness:

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8ml/min to 1.2 ml/min. Standard solution 3ppm of Clonazepam and 300ppm of Propranolol was prepared and analyzed using the varied flow rates along with method flow rate.

b) The organic composition in the mobile phase was varied from 65% to 75% standard solution 3 µg/ml of Clonazepam and 300 µg/ml of Propranolol were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

6. Limit of Detection

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD[7] according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOD = 3.3\ X\ \frac{\sigma}{S}}$$

Where, σ - Standard deviation (SD)

S – Slope

7. Limit of Quantification

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y- intercepts of regression[8] lines.

Formula:

$$\mathbf{LOQ = 10\ \sigma\ /Slope}$$

Where

σ - Standard deviation
S – Slope

8. Assay of Clonazepam & Propranolol in Dosage Form:

Twenty tablets/Capsules were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock arrangement (1 ml) was exchanged to five distinctive 10 ml volumetric flagons and volume was made up to 10 ml with same dissolvable framework (Mobile stage). The readied arrangements were infused in five repeats into the HPLC framework and the perceptions were recorded.

Forced Degradation Studies

The API (Clonazepam and Propranolol) was subjected to keep in some stress conditions[9] in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. It is one type of accelerated stability studies of the drugs that is used to help us to determining the total fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The different types of forced degradation pathways/studies are studied here are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

1. Acid Degradation Studies:

An accurately weighed 10 mg of both the pure drug were transferred to two different clean & dry round bottom flask. 30 ml of 0.1 N HCl was added to it and it was refluxed in a water bath at 60^0C for 4 hours. Allowed to cool to room temperature. The sample was then neutralized using dilute 0.1 N NaOH solution & final concentration was prepared to 50µg/ml and 40 µg/ml for Clonazepam and Propranolol respectivelywith mobile phase. It was injected into the HPLC system against a blank of mobile phase (after optimizing the mobile phase compositions). This experiment was repeated several times using same concentration of HCl (0.1N) and observed its degradation profile. The typical chromatogram shown below is the degradation profile of Clonazepam and Propranolol in 0.1N HCl.

2. Basic Degradation Studies

An accurately weighed 10 mg of both the pure drug was transferred to two different clean & dry round bottom flask. 30 ml of 0.1N NaOH was added to it. & it was refluxed in a water bath at 60^0C for 4 hours. Allowed to cool to room temperature. The sample was than neutralized using 0.1 N HCl solution & final concentration was prepared to a mixture of 50 µg/ml and 40 µg/ml for Clonazepam and Propranolol respectively *with mobile phase. It was injected into the HPLC system against a blank of mobile phase after optimizing the mobile phase compositions. This experiment was repeated several times using same concentration of NaOH such as 0.1N to observe its degradation profile. The chromatogram shown below is the degradation profile of* Clonazepam and Propranolol *in 0.1N NaOH.*

3. Thermal Degradation Studies:

Accurately weighed 10 mg of both pure drugs were transferred to two different clean & dry round bottom flask. 30 ml of HPLC water was added to it. Then, it was refluxed in a water bath at 60^0C for 6 hours uninterruptedly. After the reflux was over, the drugs became soluble and the mixture of drugs& water was allowed to cool to room temperature final concentration was a mixture of 50µg/ml and 40µg/ml for Clonazepam and Propranolol respectivelywith mobile phase. It was injected into the HPLC system against a blank of mobile phase.

4. Photolytic Degradation Studies:

Approximately 10 mg of both the pure drug was taken in two different clean & dry Petri dish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg of the UV exposed drug was transferred to a clean & dry 10 ml volumetric flask. First the UV exposed drug was dissolved in mobile phase & final concentration was prepared toa mixture of 50µg/ml and 40µg/ml for Clonazepam and Propranolol respectively with mobile phase. Finally, this solution was injected into the HPLC system against a blank of mobile phase and chromatogram was obtained.

5. Oxidation with (3%) H_2o_2 Studies:

Accurately weighed 10 mg. of both the pure drugs were taken in two different clean & dry 100 ml volumetric flask. 30 ml of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours final concentration was prepared toa mixture of 50 µg/ml and 40 µg/ml for Clonazepam and Propranolol

respectively with mobile phase. The above sample was injected into the HPLC system.

III. RESULTS AND DISCUSSION

Method Development

Wavelength Detection

The overlay spectrum of Clonazepam and Propranolol was obtained and the isobestic point of Clonazepam and Propranolol showed absorbance's maxima at 255nm.

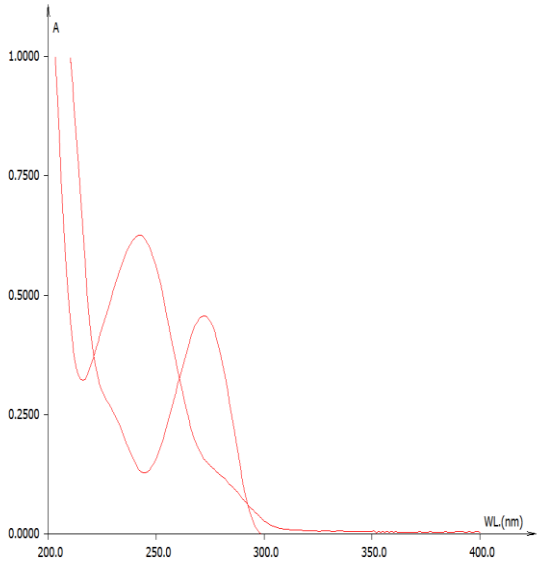

Fig.3. Overlay Spectrum for *Clonazepam* and Propranolol

Table-1: Trials and Results for the Method Development:

S.No.	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
1	Symmetry C_{18}, 5µm, 25cmx4.6mm i.d.	ACN : Water = 70 : 30	0.8 ml/min	255nm	Early elution of peak	Method rejected
2	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	Methanol: ACN = 40 :60	1.0 ml/min	255nm	Tailing Peaks	Method rejected
3	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	ACN: Phosphate buffer (0.02M) = 70:30	1.0 ml/min	255nm	Low resolution peak	Method rejected
4	Develosil ODS HG-5 RP C_{18}, 5µm,15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.01M) = 50:50 (pH-3.8)	1.0 ml/ min	255nm	Many Peaks	Method rejected
5	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.02M) = 65:35 (pH-2.6)	1.0 ml/min	255nm	Many Peaks	Method rejected
6	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.	Methanol : Phosphate buffer (0.02M) = 55:45 (pH-2.6)	1.0 ml/min	255nm	Good Peaks	Method Accepted

Optimized Chromatographic Method:

Table-2: Optimized Chromatographic Conditions

Mobile phase	Methanol : Phosphate buffer (0.02M, pH-2.6) = 55:45%v/v
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.

Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

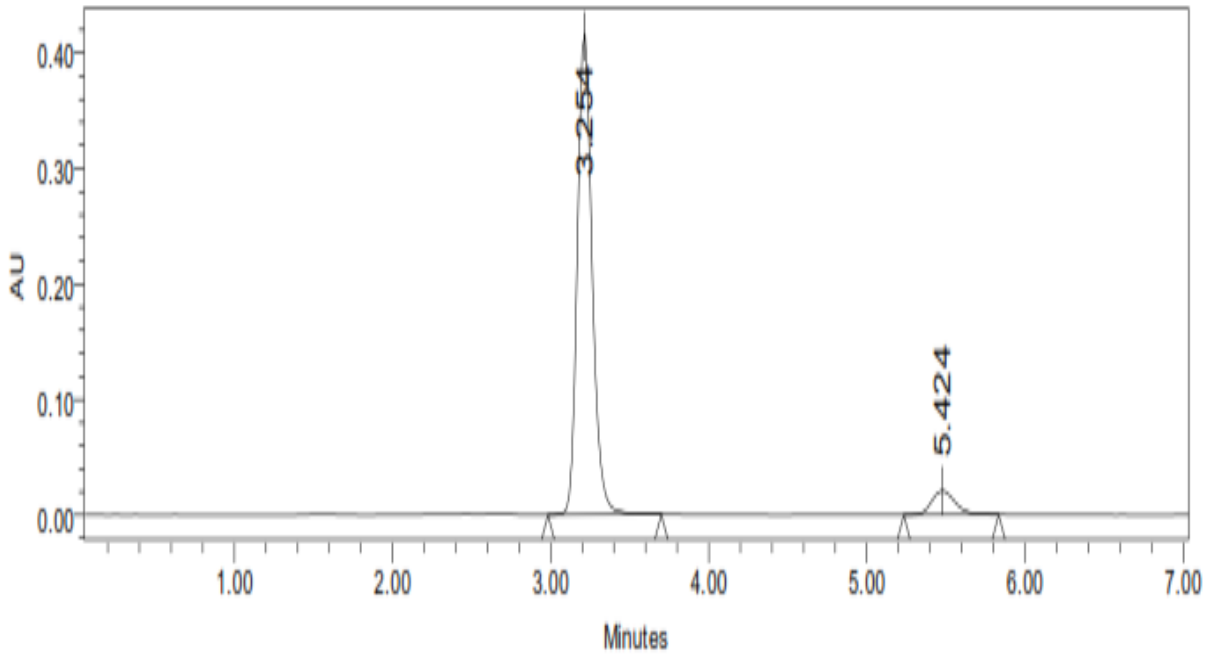

Fig.4. Optimized Chromatographic Condition

Method Validation

1. System Suitability: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system[10] that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-3.

Table-3: System suitability results for Clonazepam and Propranolol (Flow rate)

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	3.57
2	Asymmetry	T ≤ 2	Clonazepam = 0.12 Propranolol = 0.24
3	Theoretical plate	N > 2000	Clonazepam = 2987 Propranolol = 3014

2. Linearity: To evaluate the linearity[11], serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 0-14µg/ml for Clonazepam and concentration ranging from 0-28µg/ml for Propranolol. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[12] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

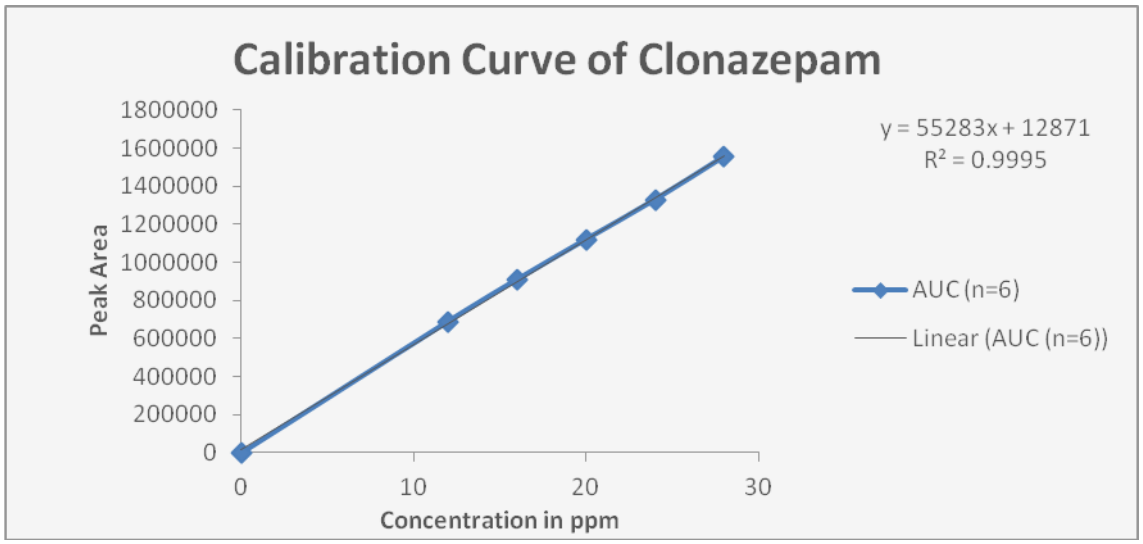

Fig.5. Standard curve for Clonazepam

Table-4: Linearity Readings for Clonazepam

CONC. (µg/ml)	MEAN AUC (n=6)
0	0
6	192164
8	247293
10	306089
12	370481
14	447930

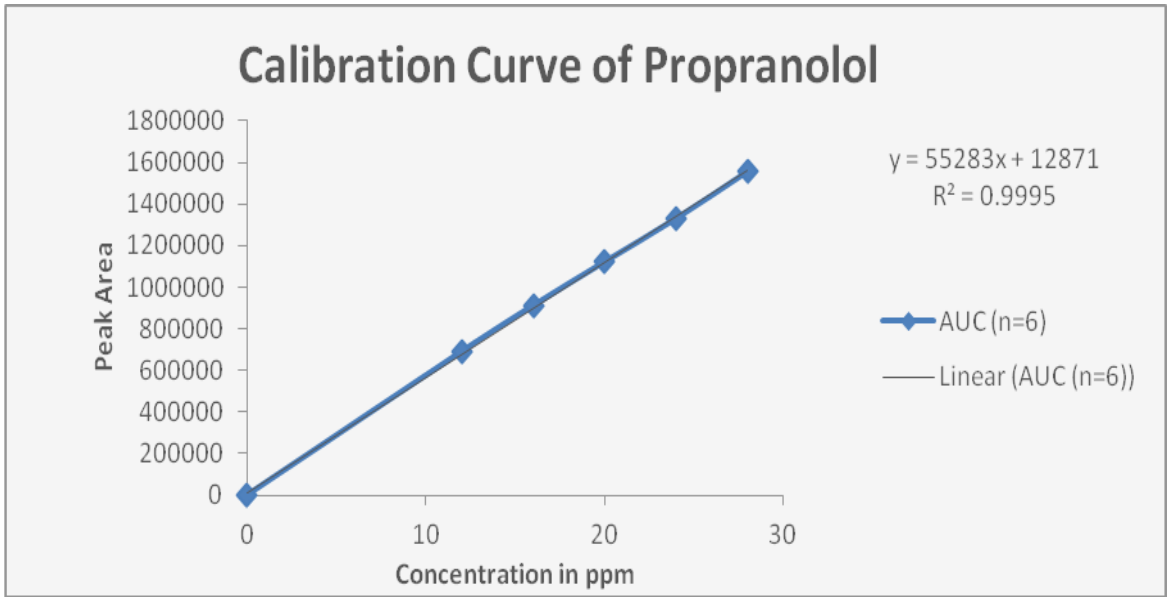

Fig.6. Standard curve for Propranolol

Table-5: Linearity Readings for Propranolol

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Observation: Linearity range was found to be 0-14 µg/ml for Clonazepam. The correlation coefficient was found to be 0.999, the slope was found to be 55283 and intercept was found to be 12871 for Clonazepam. Linearity range was found to be 0-28 µg/ml for Propranolol. The correlation coefficient was found to be 0.999, the slope was found to be 55283 and intercept was found to be 12871 for Propranolol.

3. Accuracy:

Clonazepam and Propranolol standard stock solution of 10mg/mL & 20mg/mL was used to prepare 8, 10, 12 µg/mL & 16, 20, 24 µg/mL concentrations and injected for the accuracy studies. The area under curve obtained was checked and analyzed for the recovery percentage[13].

Table-6: Accuracy results of Clonazepam

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.064107	458679	99.867	Mean= 100.4113% S.D. = 0.473694346 % R.S.D.= 0.471753
S_2 : 80 %	8	7.843532	446485	100.637	
S_3 : 80 %	8	8.19449	465887	100.73	
S_4 : 100 %	10	9.892661	559767	99.41	Mean= 100.6646667% S.D. = 1.166369295 R.S.D.= 1.158667
S_5 : 100 %	10	9.978655	564521	100.868	
S_6 : 100 %	10	10.19623	576549	101.716	
S_7 : 120 %	12	11.85907	668476	99.878	Mean= 100.4637% S.D. = 0.51154309 % R.S.D. = 0.509181
S_8 : 120 %	12	12.16785	685546	100.69	
S_9 : 120 %	12	12.18644	686574	100.823	

Table-7: Accuracy results of Propranolol

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	15.71861	881843	98.24132	Mean= 98.66425667% S.D. = 0.558426265% R.S.D.= 0.565996
S_2 : 80 %	16	15.75267	883726	98.4542	
S_3 : 80 %	16	15.88756	891183	99.29725	
S_4 : 100 %	20	20.00427	1118767	100.0213	Mean= 100.8802% S.D. = 0.945972362 % R.S.D.= 0.9377182
S_5 : 100 %	20	20.37881	1139473	101.8941	
S_6 : 100 %	20	20.14504	1126549	100.7252	

S_7 : 120 %	24	23.69705	1322915	98.73771	Mean= 98.87614%
S_8 : 120 %	24	23.73053	1324766	98.87722	S.D. = 0.137893172
S_9 : 120 %	24	23.76324	1326574	99.01349	% R.S.D. = 1.401528

Observation: The mean recoveries were found to be 100.411, 100.664 and 100.463% for Clonazepam and 98.664, 100.880 and 98.876% for Propranolol. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

4. Precision: The precision[14] of each method was ascertained separately from the peak areas obtained by actual determination of five replicates of a fixed amount of drugs Clonazepam & Propranolol. The percent relative standard deviations were calculated for Clonazepam & Propranolol are presented in the Table-8.

i) Repeatability

Table-8: Repeatability Results of Clonazepam and Propranolol

HPLC Injection Replicates	AUC for Clonazepam	AUC for Propranolol
Replicate – 1	623568	1113214
Replicate – 2	613241	1105241
Replicate – 3	625408	1113424
Replicate – 4	617412	1105987
Replicate – 5	612541	1104216
Replicate – 6	622546	1113219
Average	**615786**	**1109216.833**
Standard Deviation	**5510.431332**	**4493.157884**
% RSD	**0.890043**	**0.405074**

Observation: The repeatability study which was conducted on the solution having the concentration of about 10µg/ml for Clonazepam and 20µg/ml for Propranolol (n =5) showed a RSD of 0.890043% for Clonazepam and 0.405074% for Propranolol. It was concluded that the analytical technique showed good repeatability[15].

ii) Intermediate precision / Ruggedness

Table-9: Ruggedness Results for Clonazepam

Conc. of Clonazepam (API) (µg/ml)	Observed Conc. of Clonazepam (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.21	0.76	8.23	0.46
10	10.37	0.33	10.36	0.57
12	12.56	0.23	12.56	0.75

Table-10: Ruggedness Results for Propranolol

Conc. Of Propranolol (API) (µg/ml)	Observed Conc. of Propranolol (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
16	16.12	0.65	16.34	0.55
20	20.43	0.54	20.67	0.27
24	24.33	0.76	24.37	0.51

Observation: Intraday and interday studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

5. Robustness: Robustness is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness[16] of a method is done by varying the chromatographic parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method.

Table-11: Result of Method Robustness Test for Clonazepam

Change in parameter	% RSD
Flow (0.8 ml/min)	0.55
Flow (1.2 ml/min)	0.86
More Organic	0.88
Less Organic	0.81
Wavelength of Detection (261 nm)	0.81
Wavelength of detection (257 nm)	0.79

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Clonazepam (API).

Table-12: Result of Method Robustness Test for Propranolol

Change in parameter	% RSD
Flow (0.8 ml/min)	1.03
Flow (1.2 ml/min)	0.68
More Organic	0.77
Less Organic	0.63
Wavelength of Detection (233 nm)	1.09
Wavelength of detection (229 nm)	0.92

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Propranolol (API).

6. LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.

$$L.O.D. = 3.3 (SD/S).$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOD was found to be 0.06 µg/ml and 0.09 µg/ml for Clonazepam and Propranolol respectively.

7. LOQ: The Limit of Quantitation[17] (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ.

$$L.O.Q. = 10 (SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOQ was found to be 0.18 µg/ml and 0.27 µg/ml for Clonazepam and Propranolol respectively.

8. Assay: – Assay[18] refers to chromatography based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity.

$$\text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

The assay was performed as explained in the previous chapter. The results which are obtained are following:

Table-13: Assay of CLONAZEPAM & PROPRANOLOL Tablets

This Combination is not Available	Labelled amount of Drug (mg) Clonazepam /Propranolol	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Petril Beta-10 (Micro Labs Ltd)	0.25/10	0.198 (±0.56)/9.687 (±0.63)	99.2 (±0.284)/ 99.75 (±0.396)

Results and Discussion: The assay of Petril Beta-10 Tablets containing Clonazepam was found to be 99.2 % and Propranolol was found to be 99.75 %.

Forced Degradation Studies

Results of degradation studies:

The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Clonazepam and Propranolol were stable only in basic and thermal, oxidation stress conditions and photolytic stress conditions. The results of stability studies are given in the following Table-14.

Table-14: Results of Force Degradation Studies of Clonazepam and Propranolol API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

IV. SUMMARY AND CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Clonazepam and Propranolol in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Clonazepam and Propranolol indicated that the developed method is specific for the simultaneous estimation of Clonazepam and Propranolol in the bulk a*nd pharmaceutical dosage forms.*Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The specific Retention times for Clonazepam and Propranolol are found to be 3.254 and 5.424. The tailing factors were found to be 1.14 and 1.22 with theoretical plates 2236 and 2762 for Clonazepam and Propranolol respectively. The %Recoveries was determined as 100.512% and 99.473% for Clonazepam and Propranolol in Accuracy. The %RSD in Repeatability is 0.89 and 0.40 with Intermediate Precision are 0.44 and 0.65 for Clonazepam and Propranolol in Precision. In

Linearity, the correlation coefficient was found to be 0.999 and 0.999 for Clonazepam and Propranolol. The LOD for Clonazepam and Propranolol was 0.06 and 0.09 and LOQ for Clonazepam and Propranolol are 0.18 and 0.27

REFERENCES

1. https://go.drugbank.com/drugs/DB01068
2. https://pubchem.ncbi.nlm.nih.gov/compound/Clonazepam
3. https://en.wikipedia.org/wiki/Clonazepam
4. https://go.drugbank.com/drugs/DB00571
5. https://pubchem.ncbi.nlm.nih.gov/compound/Propranolol
6. https://en.wikipedia.org/wiki/Propranolol
7. L. R. Snyder, J.J. Kirkland, and J. W. Dolan, Introduction to Modern Liquid Chromatography, John Wiley & Sons, New York, 2009.
8. M.W. Dong, Modern HPLC for practicing scientists. Wiley, 2006.
9. L. R. Snyder, J.J. Kirkland, and J. L. Glajch, Practical HPLC Method Development, John Wiley & Sons, New York, 1997.
10. S. Ahuja and H. T. Rasmussen (Ed), HPLC Method Development for Pharmaceuticals, Academic Press, 2007.
11. S. Ahuja and M.W. Dong (Ed), Handbook of Pharmaceutical Analysis by HPLC, Elsevier/Academic Press, 2005.
12. Y. V. Kazakevich and R. Lo Brutto (ed.), HPLC for Pharmaceutical Scientists, Wiley, 2007.
13. U. D. Neue, HPLC Columns: Theory, Technology, and Practice, Wiley-VCH, New York, 1997.
14. Morgan, David J. "Fraction collector (post on Flickr)". Flickr. Retrieved 28 October 2015.
15. Karger, Barry L. (1997). "HPLC: Early and Recent Perspectives". Journal of Chemical Education. 74: 45.
16. Henry, Richard A. (1 February 2009) "The Early Days of HPLC at Dupont". Chromatography Online. Avanstar Communications Inc.
17. Karger, B. L.; Berry, L. V. (1971). "Rapid liquid-chromatographic separation of steroids on columns heavily loaded with stationary phase". Clin. Chem. 17 (8): 757–64. PMID 4254537.
18. Giddings, J. Calvin (1965) Dynamics of Chromatography, Part I. Principles and Theory. Marcel Dekker, Inc., New York. p. 281.
19. D. Meghana1*, K. Lahari1, K. Shantha Kumari1, K. Prakash1, Development and Validation of RP-HPLC Method for Simultaneous Estimation of Clonazepam and Propranolol Hydrochloride in Bulk and Pharmaceutical Dosage Forms, Inventi Rapid: Pharm Analysis & Quality Assurance Vol. 2012, Issue 4, Pages: 1-4.
20. Payal Kate1*, Parag Patel1 , Nikita Patel1 , Gaurav Kulkarni2 , Bhawan kumar Patel3, Stability indicating RP-HPLC method development and validation of Etizolam and Propranolol hydrochloride in pharmaceutical dosage form, World Journal of Pharmaceutical Sciences, 2015; 3(6): 1113-1124.
21. Umadurai M and Vijaya Nagarajan, Development and validation of a rapid UPLC Assay method for the simultaneous estimation of paroxetine and clonazepam in tablet dosage form, International Journal of Chemical and Pharmaceutical Sciences 2014, Dec., Vol. 5 (4), Pages: 42-47.