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Development and Validation of analytical method for the simultaneous estimation of Clonazepam and Propranolol by using RP-HPLC

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ABSTRACT: Background: The recent analytical research is significantly involved in the method development for API drugs by RP-HPLC. **Aim:** A novel, precise, accurate, rapid and cost effective isocratic reverse phase high performance liquid chromatographic (RP-HPLC) method was developed, optimized and validated for the estimation of Clonazepam (CLO) and Propranolol (PRO) in bulk and pharmaceutical dosage forms. **Method:** The drugs were estimated using Phenomenex Gemini C18 (4.6 × 150 mm, 5.0 µm) particle size column. A mobile phase composed of tri ethylamine buffer and methanol in proportion of 32: 68 v/v, at a flow rate of 1.0 ml/min was used for the separation. Detection was carried out at 248 nm. **Results:** The linearity range obtained was 30 to 70 µg/ml for Clonazepam and 10 to 50 µg/ml for Propranolol. The retention times (Rt) of 3.297 and 5.405 min for Clonazepam and Propranolol respectively. The correlation coefficient values were found to be 0.999 and 0.999. Precession studies showed % RSD values less than 2 % for both the drugs in all the selected concentrations. The percentage recoveries of Clonazepam (CLO) and Propranolol (PRO) were found to be 100.1873 % for Clonazepam and 100.748 % for Propranolol respectively. The assay results of Clonazepam (CLO) and Propranolol (PRO) were found to be 99.82 %. The limit of detection (LOD) and limit of quantification (LOQ) were 2.6 and 7.8µg/ml for Clonazepam and 3.4 and 10.2µg/ml for Propranolol respectively. **Conclusion:** The proposed validated method as per ICH was successfully used for the quantitative analysis of commercially available dosage form.

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INTRODUCTION:

Analytical chemistry1 is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation [1]. To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the

Clonazepam (CLO) and Propranolol (PROP) in dosage form. Clonazepam, a benzodiazepine, is used primarily as an anticonvulsant in the treatment of absence seizures, petit mal variant seizures (Lennox-Gastaut syndrome), akinetic and myoclonic seizures, and nocturnal myoclonus [2]. It enhances the activity of gamma aminobutyric acid (GABA), which is a major inhibitory neurotransmitter in the central nervous system. In animals, convulsions are antagonized occurs following administration of clonazepam [3]. The primary objective of proposed work is to develop new simple, sensitive, accurate and economical analytical method for the simultaneous estimation of Clonazepam (CLO) and Propranolol (PROP).

MATERIALS AND METHODS:

Materials:

The methanol and tri ethylamine buffer were purchased from S.D. Fine chemical, Mumbai. The clonazepam and propranolol tablets were purchased from local market of Moinabad. Clonazepam and propranolol pure drug was procured as gift sample from Pharmaceutical Company. All other chemicals and reagents used in this study are of analytical grade and were procured from an authorized dealer.

Table 1. HPLC method development protocol.

Parameters	Specified data
Instrument used	Waters HPLC with auto sampler and PDA detector 996 model
Temperature	38 ˚C
pH	4.8
Column	Phenomenex Gemini C18 (4.6 × 150 mm, 5.0 µm) particle size
Mobile phase	Methanol: TEA buffer pH 4.8 (32:68 v/v)
Flow rate	1 ml/ min
Wavelength	248 nm
Injection volume	20 µl
Run time	7 min

HPLC method development [4-6]:

***Preparation of standard solution*:**

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of methanol was added. The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same methanol. Further 2.25 ml of the above Clonazepam and 0.45 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with methanol.

***Procedure*:**

The samples were injected by changing the chromatographic conditions and the chromatograms were recorded. The conditions of proper peak elution were noted for performing validation parameters as per ICH guidelines.

Validation method [7,8]:

***Preparation of mobile phase*:**

Accurately 320 ml (32 %) of HPLC methanol and 680 ml of TEA buffer (68 %) were measured and mixed. The mixture was degassed in a digital ultra sonicater for 15 min and then the mixture was filtered through 0.45 µ filter under vacuum filtration.

***Diluent Preparation*:**

The mobile phase was used as the diluent.

Validation parameters (System Suitability) [9,10]:

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of methanol was added. The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same methanol. Further 0.5 ml of the above Clonazepam and 0.3 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with methanol.

Procedure:

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

Specificity study of drug [11]:

***Preparation of standard solution*:**

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of diluent was added. The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same solvent. Further 0.5 ml of the above Clonazepam and 0.3 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with diluent.

***Preparation of sample solution*:**

The average weight of ten Tablets was taken and crushed in a mortar by using pestle. The powder weight equivalent to 10 mg of Clonazepam and Propranolol sample was taken into a 10 ml clean dry volumetric flask. To the flask about 7 ml of diluents was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent. Further 0.5 ml of Clonazepam and 0.3 ml of Propranolol was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***Procedure*:**

The three replicate injections of standard and sample solutions were injected and calculate the assay by using formula:

$$\text{Assay (\%)} = \frac{(SA \times WS \times DS \times P \times WT)}{(StA \times DOS \times WOS \times LC)} \times 100 \quad \ldots..(1)$$

Where SA and StA are sample and standard area. WS and DOS are weight and dilution of standard. P is purity. WT and WOS are weight of tablet and of sample. LC is label claim.

***For preparation of 50 % Standard stock solution*:**

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of diluent was added. The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same solvent. Further 0.25 ml of the above Clonazepam and 0.15 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with diluent.

***For preparation of 100 % Standard stock solution*:**

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of diluent was added.

The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same solvent. Further 0.5 ml of the above Clonazepam and 0.3 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with diluents.

***For preparation of 150 % Standard stock solution*:**

Accurately 10 mg of Clonazepam and Propranolol working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To the flask, about 7 ml of diluents was added. The mixture was sonicated to dissolve and removal of air completely. The volume was made up to the mark with the same solvent. Further 0.75 ml of the above Clonazepam and 0.45 ml of the Propranolol stock solutions were pipetted into a 10 ml volumetric flask and diluted up to the mark with diluents.

RESULTS AND DISCUSSION:

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Clonazepam and Propranolol was done by RP-HPLC. The TEA buffer was pH 4.8 and the mobile phase was optimized with consists of methanol: TEA buffer mixed in the ratio of 32:68 % v/ v. A Phenomenex Gemini C18 (4.6 × 150 mm, 5.0 µm) particle size or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min [12].

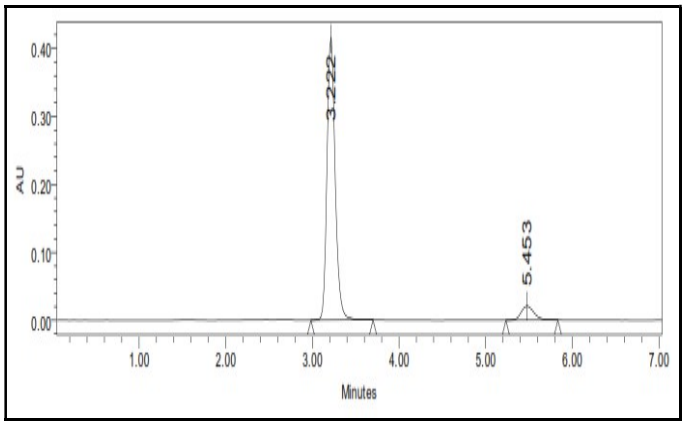

Fig 1. Optimized Chromatogram of drug sample.

From the above chromatogram it was observed that the Clonazepam and Propranolol peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Acceptance criteria are Resolution between two drugs must be not less than 2, Theoretical plates must be not less than 2000 and tailing factor must be not less than 0.9 and not more than 2. It was found from above data that all the system suitability parameters for developed method were within the limit [13,14].

The acceptance criteria that are % RSD of five different sample solutions should not more than 2 [15]. The % RSD obtained is within the limit, hence the method is suitable.

The % purity of Clonazepam and Propranolol in pharmaceutical dosage form was found to be 99.82 %.

The linearity range of Clonazepam and Propranolol were found to be from 30 to 70 and 10 to 50 µg/ml

Table 2. Optimized Chromatogram data of Clonazepam and Propranolol (Standard).

Sl. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Clonazepam	3.297	859856	42569	1.24	7896	--
2	Propranolol	5.405	5698	3652	1.36	6582	6.8

RT – Retention time.

Table 3. Optimized Chromatogram data of sample drug.

Sl. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Clonazepam	3.222	865898	43659	1.26	7985	--
2	Propranolol	5.453	5789	3785	1.38	6659	6.8

Table 4. Results of system suitability for Clonazepam.

Sl. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Clonazepam	3.200	859865	42568	7895	1.24
2	Clonazepam	3.248	859788	42587	7859	1.24
3	Clonazepam	3.299	857984	42659	7869	1.24
4	Clonazepam	3.297	854879	42875	7849	1.24
5	Clonazepam	3.297	857896	42487	7859	1.23
Mean	--	--	858082.4	--	--	--
Std. Dev.	--	--	2024.409	--	--	--
% RSD	--	--	0.235922	--	--	--

Table 5. Results of System Suitability for Propranolol.

Sl. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Propranolol	5.413	5689	3659	6583	1.36
2	Propranolol	5.484	5687	3648	6592	1.37
3	Propranolol	5.405	5682	3698	6549	1.37
4	Propranolol	5.405	5649	3675	6571	1.36
5	Propranolol	5.409	5674	3649	6529	1.36
Mean	--	--	5676.2	--	--	--
Std. Dev.	--	--	16.2696	--	--	--
% RSD	--	--	0.286628	--	--	--

Table 6. Peak results for assay sample Clonazepam.

Sl. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Clonazepam	3.297	865985	43659	1.26	7985
2	Clonazepam	3.294	865798	43875	1.26	7925
3	Clonazepam	3.295	865456	43659	1.27	7946

Table 7. Peak results for assay sample Propranolol.

Sl. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Propranolol	5.435	5789	3659	1.37	6659	6.9
2	Propranolol	5.417	5798	3684	1.38	6689	7.0
3	Propranolol	5.434	5749	3695	1.38	6648	6.9

respectively. Linear regression coefficient was not more than 0.999, 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98 to 102 % of Clonazepam and Propranolol. LOD and LOQ were found to be within limit.

CONCLUSION:

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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