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DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE DETERMINATION OF CLONOZEPAM AND RELATED IMPURITIES IN A PHARMACEUTICAL FORMULATION

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ABSTRACT
The present study describes a simple reverse phase HPLC method for the determination of Clonazepam and his decomposition products present in pharmaceutical dosage form. A Superspher 60 RP-Select B, (250 × 4.0 mm, packed with 5µ) is used as stationary phase. An isocratic mode with mobile phase consisting of methanol, acetonitrile, and potassium dihydrogen phosphate buffer (KH_2PO_4) (0.05M) in ratio of (14:40:46, v/v/v) (pH* 4.1) at a flow rate of 0.9 ml/min and effluent was monitored at 242 nm. Chromatogram showed a peak of Clonazepam at retention time of 4.96 ± 0.1 min. The method was validated for linearity, accuracy, precision, limit of quantitation, limit of detection and robustness. Results indicated an excellent linearity for all the analytes over their respective concentration ranges with correlation coefficients (r^2) ≥0.999. The recovery ranged from 98.4% to 101.3% indicating a high degree of the method's accuracy. The limit of detection and limit of quantitation for estimation of Clonazepam was found to be 0.73µg/ml and 2.43µg/ml, respectively. For both intra- and inter-day coefficients of variation were less than 1.6% (R.S.D.). This showed that proposed method is rapid, simple, precise, linear, robust, and accurate which is useful and economic for routine analysis of Clonazepam in pharmaceutical dosage forms.

KEY WORDS
Clonazepam, HPLC, Reversed-phase chromatography and Validation.

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INTRODUCTION

Clonazepam (Figure No.1) 5-(2-chlorphenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepine-2-one is a benzodiazepine (BZD) drug having anxiolytic, anticonvulsant, muscle relaxant, sedative, and hypnotic properties[1]. The molecular formula is $C_{15}H_{10}ClN_3O_3$ and molecular weight is 315.715. The main reason for a prescription of Clonazepam is for treatment of epilepsy and Panic Disorder. It is

approved for treatment of typical and atypical absences, infantile myoclonic, myoclonic and akinetic seizures[2] and also as a second-line agent. An open label study suggested that the combination of valproate, lamotrigine, and a benzodiazepine such as clonazepam could markedly reduce the frequency of drop attacks in patients with generalized or multifocal epilepsies[3].

Several methods for the analysis of BZDs have been reported[4]. A number of chromatographic methods, such as UV Spectroscopy[5], high-performance liquid chromatographic[6] and high-performance liquid chromatographic–mass spectrometry (LC–MS)[7,8] have been used in the analysis of clonazepam Figure No.1 and other 1,4-benzodiazopines. Several high-performance liquid chromatographic (HPLC) methods have also been reported for the determination of clonazepam and other BZDs[9,10]. However, all of these methods have limitations such as long run times and/or expensive. The present study focused on minimizing these limitations and to develop a simple precise accurate and economic method for estimation of clonazepam in tablet dosage form[11].

MATERIALS AND METHOD

Chemicals and reagents

An analytically pure sample of Clonazepam was procured as gift sample from office pharmacy (Morocco). Acetonitrile and methanol (HPLC grade) were procured from Merck Specialist. Ultra pure water (HPLC-grade) was obtained from Merck. Potassium dihydrogen phosphate (AR grade, purity 99.6%) was procured from Merck. Tablet formulations were procured from a local pharmacy with labeled amount 2 mg per tablet. Other chemicals used were of analytical grade.

Instrumentation and chromatographic conditions

The HPLC system used for quantification of Clonazepam consisted of a LaChrom L-7100 Merck Hitachi Pump, LaChrom L-7200 Merck Hitachi Autosampler and LaChrom L-7400 Merck Hitachi UV Detector. The chromatogram peaks were quantified by means of PC Multi- System Manager Software (Merck- Hitachi Model D-7000). Chromatography separation for analyte was achieved on Superspher 60 RP-Select B analytical column with 250 × 4.0 mm i.d. and 5 μm particle size maintained at ambient temperature. The mobile phase consisting of methanol, acetonitrile, KH_2PO_4 (0.05M) in ratio of (14:40:46, v/v/v) (pH* 4.1) that was set at a flow rate of 0.9 ml/min was found to be optimum and further optimized by adjusting pH 4.1 by adding orthophosphoric acid 85 % (m/m). The composition of methanol, acetonitrile, phosphate buffer (0.05M) in ratio of (14:40:46, v/v/v) with pH 4.1 gave the best results.

The mobile phase was degassed in an ultrasonic bath prior to use and filtered through 0.45 μm membrane filter before pumping into HPLC system. The injection volume was 20 μl, and a chromatographic peak was detected at 242 nm.

Preparation of mobile phase

Mobile phase was a mixture of 140 ml of methanol and 400ml of acetonitrile and 460ml Potassium dihydrogen phosphate 0.05M adjusted to pH 4.1 with ortho phosphoric acid 85 % (m/m).

Mobile phase was filtered through a 0.45 μm nylon filter and degassed for 5 min using an ultrasonicator.

Preparation of mixture solvent

Mixture solvent was a mixture of 140 ml of methanol and 400 ml of acetonitrile and 460 ml Potassium dihydrogen phosphate 0.05M.

Mixture solvent was filtered through a 0.45 μm nylon filter and degassed for 5 min using an ultrasonicator.

Preparation of standard solution

Accurately weighed about 100 mg of Clonazepam standard was taken in a 150 ml volumetric Brown flask and was dissolved in 20ml with Methanol.

About 125 ml diluent was added and mixture was dissolved by sonication and it was diluted up to mark with Mixture solvent. 10 ml of this solution was further diluted to 50 ml with Mixture solvent.

Preparation of sample solution

Ten tablets of Clonazepam hydrochloride equivalent to 1500 mg of Rivotril 2 mg were weighed and were transferred into a 150 ml volumetric Brown flask and were dissolved in 20ml with Methanol. After 5 min,

125ml of mixture solvent was added and the mixture was sonicated for 10 min with intermittent shaking and then cooled at room temperature. The resulting solution was diluted with mixture solvent up to the mark. Filtered solution through 0.45 µm Teflon Syringe Filter.

After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the sample solution was loaded in the 20 µl fixed - sample loop of the injection port.

Method validation

Specificity

Specificity of proposed method was determined by checking blank and placebo interference at the retention time of Clonazepam peak. Identification of Clonazepam peak in sample solution was confirmed by comparing retention time of Clonazepam peak with retention time of solution standard of Clonazepam.

Linearity

Linearity of the method was evaluated by using 5 linearity solutions of different concentrations. Accurately measured aliquots of solution standard were taken in five different 150 ml volumetric Brown flask and diluted up to the mark with the mixture solvent such that the final concentrations of Clonazepam were 93 µg ml^{-1}, 113 µg ml^{-1}, 133 µg ml^{-1}, 153 µg ml^{-1} and 173 µg ml^{-1}. A 20 µl aliquot of each linearity solution was injected in Triplicate[11].

Accuracy

The accuracy of the method was determined by calculating recoveries of clonazepam by the standard addition method. Known amount of standard of Clonazepam was spiked to placebo in three different levels (70%, 100% and 130% of sample concentration) and prepared three spiked samples of each level (Total 9 determinations as per ICH guideline.) These spiked samples were analyzed against solution standard and the amount of Clonazepam recovered in three different levels was calculated.

Instrumental precision

The instrumental precision was checked by injecting six replicates of solution standard containing Clonazepam (133.33 µg ml^{-1}) and calculated the percentage RSD of retention time and area responses of Clonazepam.

Method precision (repeatability)

The method precision of the proposed method was determined by preparing six different sample solutions of same batch and analyzed against standard solutions. Assay values of these all six samples were calculated.

Intermediate precision (reproducibility)

The intermediate precision of the proposed method was evaluated by preparing six different sample solutions of same concentrations as prepared in method precision and analyzed against standard solutions on different days. Assay values of all the six samples were calculated.

Robustness

Robustness of method is its ability to remain unaffected by small changes in method parameters. Robustness of proposed method was demonstrated by making slight changes in method parameters like flow rate (±5%), column temperature (±2 °C), detection wavelength (±5 nm), mixture solvent composition (±5% organic phase) and used different lot of column.

Filter compatibility

To check the compatibility of filter paper used to filter sample solution, the sample solution was divided into two parts. One part of solution was centrifuged and other part of solution was filtered through different types of filter papers such as 0.45 µm PTFE syringe filter, 0.45 µm PVDF filter and 0.45 µm Teflon syringe filter. Results of centrifuged sample and filtered samples were compared.

Solution stability

The solution stability of sample solution and standard solution were evaluated by comparison of assay value of freshly prepared samples and stored samples (at room temperature for 48 h). Standard solution and sample solution were prepared as mentioned in chromatographic conditions. Sample solution was analyzed and assay value was calculated against standard solution. Both the solutions (standard and sample solution) were kept at

room temperature for 24 h. After 12 h these stored samples were reanalyzed against freshly prepared standard solution and the assay values were compared.

RESULTS AND DISCUSSION

In this method to optimize chromatographic parameters several mobile phase compositions were tried. A satisfactory separation, good peak symmetry and to achieve good retention time was obtained with mobile phase consisting a mixture of methanol, acetonitrile, Potassium dihydrogen phosphate buffer (KH_2PO_4) (0.05M) in ratio of (14:40:46, v/v/v) (pH* 4.1). that was set at a flow rate of 0.9 ml/min was found to be optimum and further optimized by adjusting pH 4.1 by adding orthophosphoric acid 85 % (m/m). The suitability of the mobile phase decided on the basis of the sensitivity of the assay, time required for the analysis, ease of preparation, and use of readily available cost effective solvents. The composition of methanol, acetonitrile, Potassium dihydrogen phosphate buffer (0.05M) in ratio of (14:40:46, v/v/v) (pH* 4.1) gave the best results. The proposed method was validated as per ICH guidelines with respect to specificity, linearity, accuracy, precision, robustness, solution stability and filter paper compatibility. All results of validation parameters meet the limits of ICH guidelines. Chromatograms of blank, placebo of Clonazepam tablet and Clonazepam are shown in Figure No.2 respectively.

Specificity

It was observed that there was no interference from blank and placebo at the retention time of Clonazepam peak. Retention time of Clonazepam peak in sample solution matches the retention time of Clonazepam peak in standard solution. These results indicate that proposed method gives uniform and pure peak of Clonazepam.

Linearity

A calibration curve was obtained by plotting area response versus concentration. Correlation coefficient obtained from graph was 0.9988. Linearity curve of Clonazepam is shown in Figure No.3.

Accuracy

The percentage recoveries of Clonazepam from tablet samples were calculated. Recovery ranged between 98.39% and 101.34%. Results of recovery experiment are shown in Table No.1.

Instrumental precision

The percent relative standard deviation (RSD) for six replicate of standard solution was found to be 0.28% and 1.27% for retention time and area response respectively. Results are shown in Table No.2 and Chromatograms of six injections of standard solutions of Clonazepam are shown in Figure No.4 respectively.

Method precision

Percent relative standard deviation (RSD) of Assay values for six samples were found to be 1.38%. The low RSD values indicate that the proposed method is precise or repeatable.

Intermediate precision or reproducibility

% RSD of assay values of 12 samples (method and intermediate precision sample) were found to be 1.54%. The closeness of assay results and percent RSD values indicate that the proposed method is reproducible.

LOD and LOQ

LOD and LOQ for Clonazepam were estimated by injecting a series of dilute solutions with known concentration. The parameters LOD and LOQ were determined on the basis of peak response and slope of the regression equation. The LOD and LOQ of the drug were found to be 0.728µg/ml and 2.43µg/ml respectively.

Robustness

It was observed that by making changes in chromatographic parameters, absolute difference between percent assay under altered condition and mean percent assay obtained during repeatability was not more than 2.0%. % RSD of area response and retention time were below 2%. The results of Robustness evaluation are shown in Table No.3.

Filter compatibility

The percent assay values were calculated for centrifuged and filtered samples. The results obtained using filter papers were compared with results obtained with centrifuged sample. Absolute

difference between results for filtered solutions and centrifuged solutions was not more than 2.0%. It was observed that filter paper does not adsorb drug substance during filtration of sample solutions.

Solution stability

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 48 h at an interval of 12 h at room temperature. The results show that for solutions, the retention time and peak area of clonazepam hydrochloride remained unchanged and no significant degradation within the indicated period, this indicates that both solutions were stable for 24h.

Table No.1: Recovery results of Clonazepam

S.No	Accuracy level	Sample preparations	Added amount of Clonazepam (mg ml^{-1})	Recovered amount of Clonazepam (mg ml^{-1})	% Recovery	Mean % recovery	% RSD
1	Accuracy (70%)	Preparation-1	0.0933	0.0918	98.39	98.55	0.14
		Preparation-2	0.0964	0.0951	98.65		
		Preparation-3	0.0938	0.0925	98.61		
2	Accuracy (100%)	Preparation-1	0.1341	0.1359	101.34	100.65	1.14
		Preparation-2	0.1337	0.1354	101.27		
		Preparation-3	0.1334	0.1325	99.33		
3	Accuracy (130%)	Preparation-1	0.1732	0.1726	99.65	99,40	0.61
		Preparation-2	0.1778	0.1755	98.71		
		Preparation-3	0.1772	0.1769	99,83		

Table No.2: Instrumental precision results of Clonozepam

S.No	Concentration (μg/ml)	Retention time (min)	Area response
1 2 3	138.26 138.26 138.26	4.96	100541
		4.98	101571
		5.00	99751
4 5 6	138.26 138.26 138.26	4.99	102515
		4.99	103321
		4.99	101529
Mean % RSD		4.985	101538
		0.28	1.27

Table No.3: Results of robustness study

S.No	Method parameter	Altered condition	% Assay	% RSD
1	Flow rate	0.855 ml min^{-1}	98.85	1.47
		0.90 ml min^{-1}	99.00	1.38
		0.945 ml min^{-1}	100.23	0.93
2	Temperature	23 °C	99.31	0.81
		25 °C	99,00	1.38
		27 °C	99.98	0.58
3	Wavelength (nm)	237 nm	98.52	0.83
		242 nm	99.00	1.38
		247 nm	100.34	0.43
4	Mobile phase composition (Methanol: Acetonitrile: KH_2PO_4 (0.05M) (v/v/v) (pH* 4.1). Columns	16:38:46	98.63	0.62
		14:40:46	99.00	1.38
		12:42:46	99.71	0.79
		Lot-1	99.00	1.38
		Lot-2	99.75	1.13

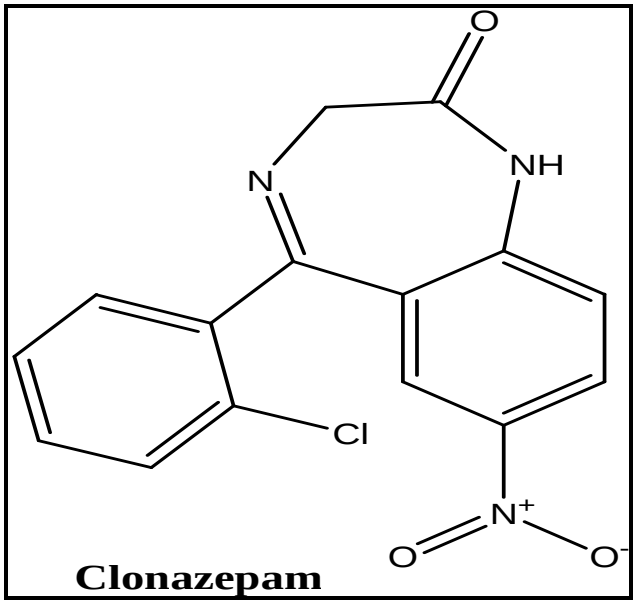
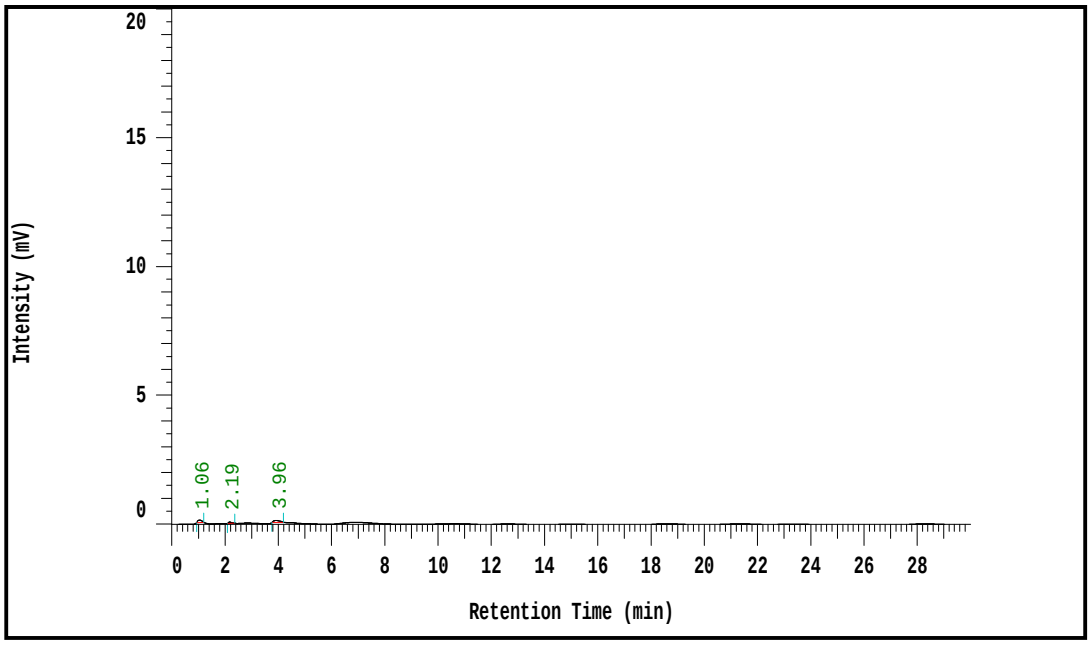
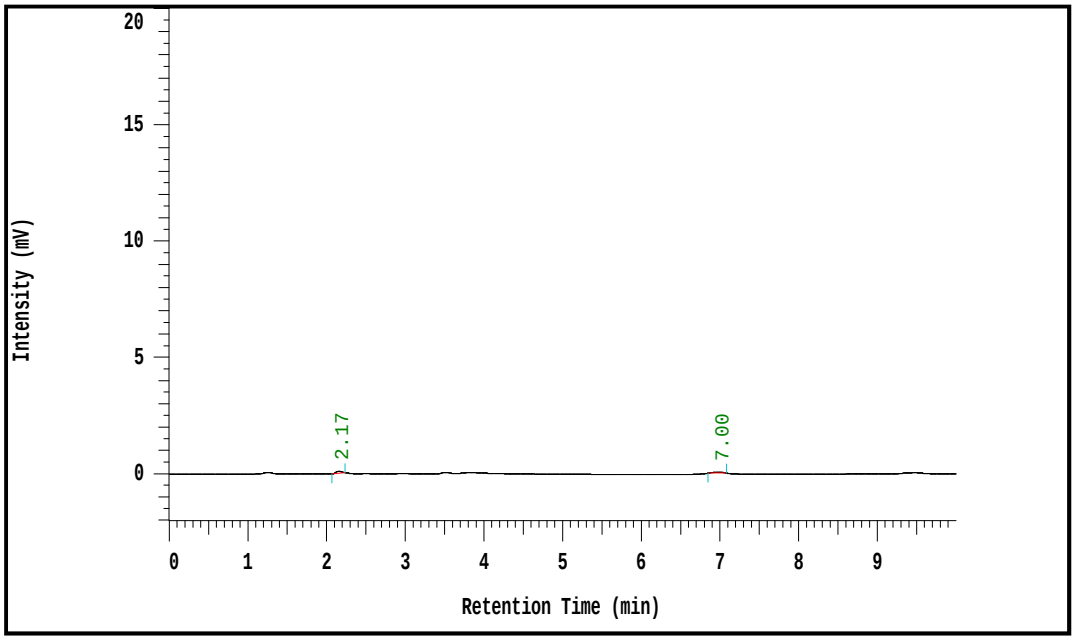


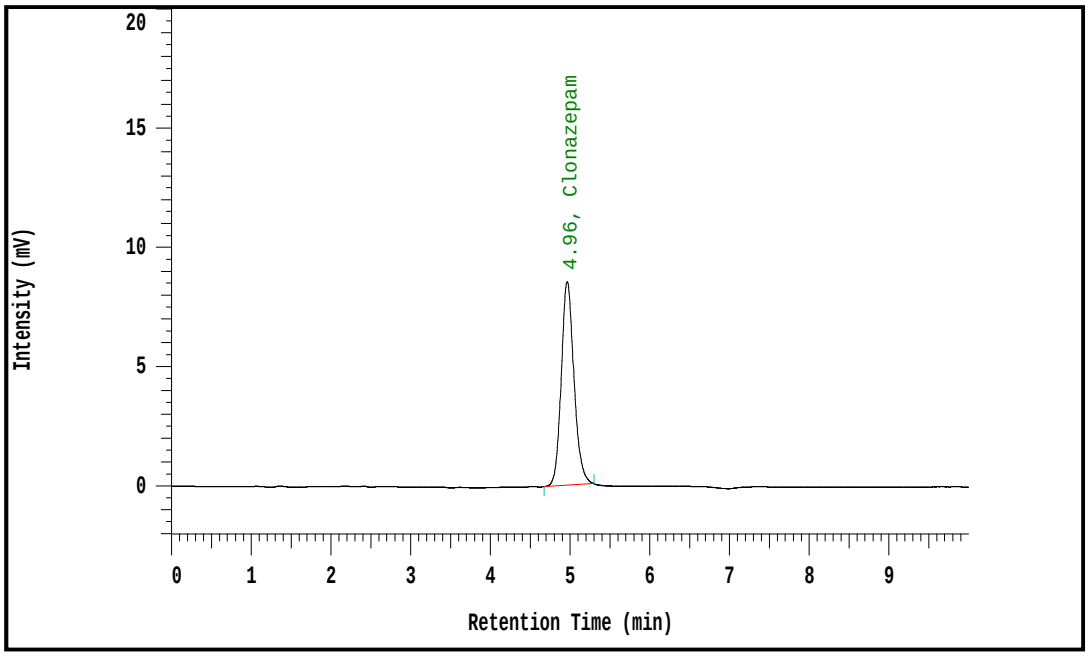
Figure No.1: Molecular structure of clonazepam



(2a) Chromatogram of blank



(2b) Chromatogram of placebo of Clonazepam tablet



(2c) Chromatogram of Clonazepam

Retention time of Clonazepam is 4.96 ± 0.1 min.

Figure No.2: Chromatograms of blank (2a), placebo of clonazepam tablet (2b) and clonazepam (2c)

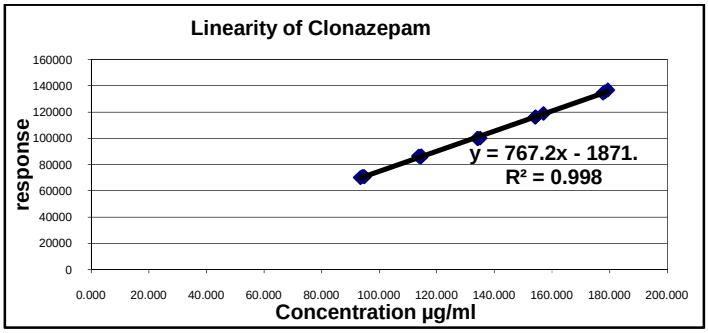


Figure No.3: Linearity curve of clonazepam

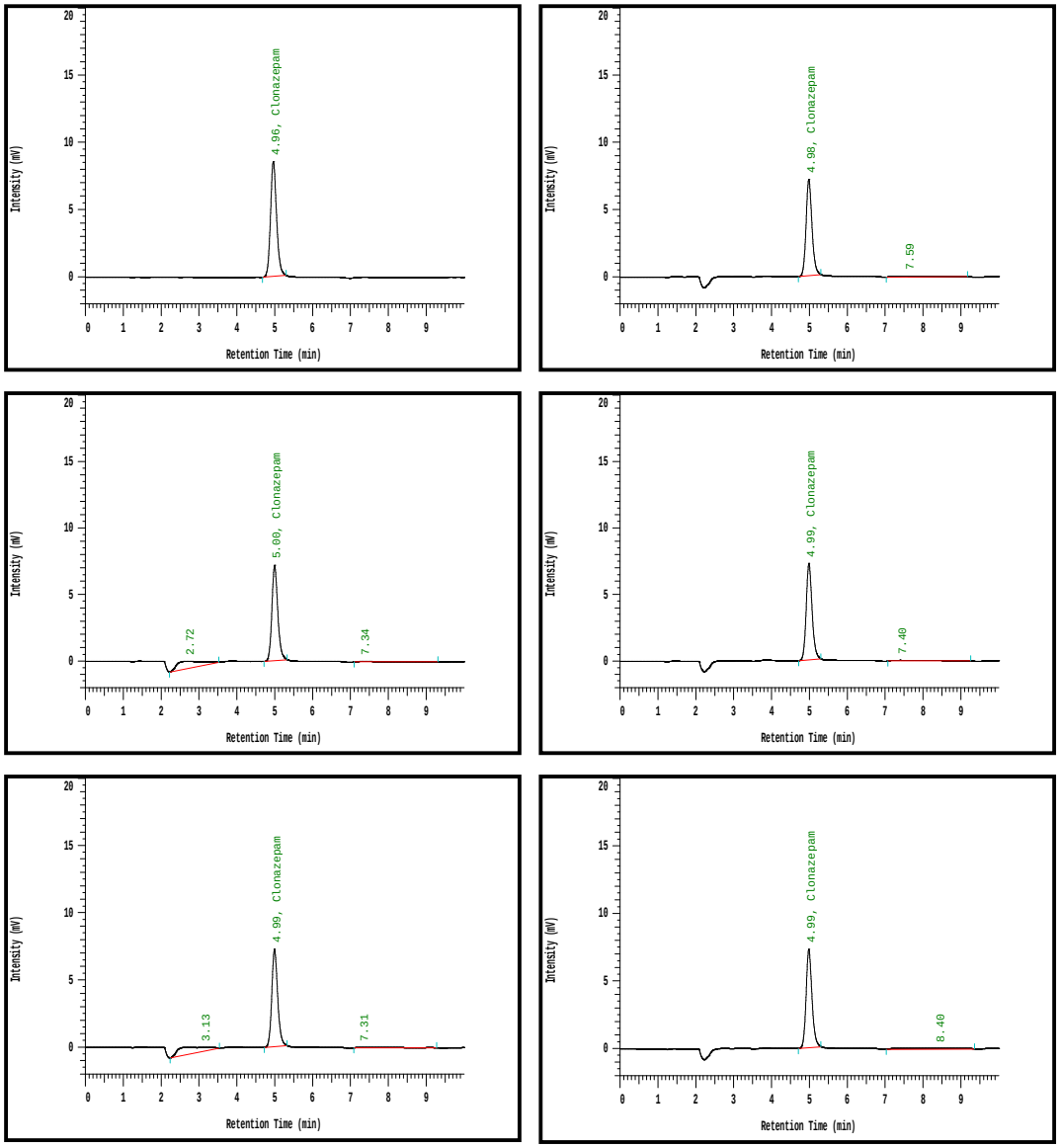


Figure No.4: Chromatograms of six injections of standard solutions of Clonazepam

CONCLUSION

A convenient, rapid, accurate, precise and economical RP-HPLC method has been developed for estimation of Clonazepam in bulk and tablet dosage form. The assay provides a linear response across a wide range of concentrations and it utilizes a mixture solvent which can be easily prepared and diluent is economic, readily available. The proposed method can be used for the routine analysis of Clonazepam hydrochloride in bulk preparations of the drug and related impurities, in pharmaceutical dosage forms without interference of excipients.

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