International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

DEVELOPMENT OF LIQUID CHROMATOGRAPHIC AND VISIBLE SPECTROPHOTOMETRIC METHODS FOR THE ESTIMATION OF CLOBAZAM IN TABLET DOSAGE FORMS

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ABSTRACT

An isocratic RP-HPLC method (Method-A) and two visible spectrophotometric methods (Method B and C) were developed and validated for the determination of Clobazam in bulk and tablet dosage forms. In Method-A, a PEAK chromatographic system equipped with Zodiac C-18 (250mm, 4.6mm and 5µm) column, mobile phase of composition 10:30:60 (v/v/v) of tetra hydro furan (THF), methanol (MeOH) and acetonitrile (ACN), UV detector were adopted for the chromatographic analysis. The output signal was monitored at a detection wavelength at 227 nm and integrated by PEAK Chromatographic Software version 1.06. In spectrophotometric methods, the chromogenic reagents such as Fe (II)-1, 10 phenanthroline (Methods-B) and ferricyanide-Fe (III) (Method-C) reagents were used for color development for the estimation of Clobazam in pure and formulations. The developed methods were validated according ICH guidelines and adopted for the assay of clobazam in the bulk drug and formulations.

Keywords: Clobazam, RP-HPLC method, Ferricyanide , Phenanthroline, Validation, Assay

INTRODUCTION

Clobazam [CBZ] is a 1,5-benzodiazepine, meaning that its diazepine ring has nitrogen atoms at the 1 and 5 positions instead of the usual 1 and 4. Like other 1, 5-benzodiazepines such as arfendazam, lofendazam, it has less affinity for the ω_1-allosteric binding site on the $GABA_A$ receptor compared to the 1,4-benzodiazepines. CBZ has selective affinity for the ω_2 site, where it has agonistic activity. It is marketed as anxiolytic and anticovulsant and used for the treatment of various seizure types and epilepsy [1]. CBZ is chemically known as 7-chloro-1-methyl-5-phenyl-1, 5-benzodiazepine-2, 4 (3H-dione) and its molecular structurer is represented by Figure-1. The molecular formula and molecular weight are x and y respectively. CBZ is available in oral form under the brand names Frisium, Urbanol and Onfi.

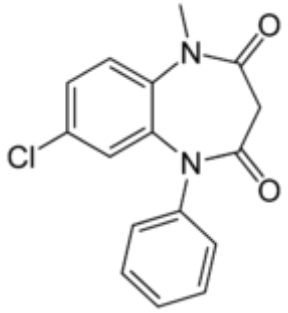

Figure-1: Molecular structure of Clobazam

An extensive literature survey was carried out and found one UV spectrophotometry [2] method for the estimation of clobazam. A few HPLC methods were available for the determination of the clobazam in different biological fluids [3-6] and pharmaceutical formulations [7-8]. Some simultaneous RP-HPLC methods [9, 10] determination methods for the assay of clobazam and its metabolites in human fluids and a stability indicating HPLC method [11] for the determination and characterization of basic degradation product of clobazam were also reported.

MATERIALS AND METHODS

Clobazam reference standard was provided by Reyan Pvt. Ltd., formulation tablets were purchased from a local pharmacy (Med Plus). HPLC grades methanol (MeOH), acetonitrile (ACN), tetra hydro furan (THF) and tri ethyl amine (TEA) were purchased from Merck Specialities Pvt. Ltd., Mumbai. All other chemicals and reagents such as potassium ferricyanide, ferric chloride and 1, 10 phenanthroline were of AR grade and were purchased from Qualigens Fine Chemicals, Mumbai.

Instrumentation: The PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 Techcomp was used Method-A. The output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. In Method-B and C, a UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Denver electronic analytical balance (SI-234) and Systronics digital pH meter were used for weighing and to adjust pH of the mobile phase.

Preparation of solutions: In Method-A, an amount of the pure drug equivalent to 10mg of Clobazam was accurately weighed and transferred into a 10ml volumetric flask, dissolved with 10ml of mobile phase, sonicated for five minutes and made up to the mark with mobile phase. The solution was filtered through Ultipor N_{66} Nylon 6, 6 membrane sample filter paper. To prepare sample solution, the average weight of five tablets of Clobazam (Frisium -20 mg) was determined and powdered them with mortar. An amount of powder equivalent to 10mg of Clobazam was weighed accurately and transferred into a clean 10 ml volumetric flask and dissolved in mobile phase solution. Then the solution was made up to the volume with mobile phase. The solution was sonicated for 5min and filtered through 0.45 µm membrane filter. Then about 5.0ml of the stock solution of standard and sample separately transferred into two 100ml volumetric flasks and diluted up to the mark with mobile phase. The concentration of the resulting solution was found to be 50µg/ml. In similar manner, appropriate volumes of these solutions were further diluted with mobile phase to prepare solutions of required concentration. An amount of pure drug equivalent to10mg was accurately weighed and dissolved in 10ml of double distilled water in a standard flask. About 30.0ml or 10.0ml of this stock solution was measured and makeup to 100ml to get a concentration of 300 µg/ml or 100 µg/ml and was used as stock solution in Method-B and Method-C respectively. The sample solution was prepared by dissolving an amount of the powder (Frisium -20 mg) equivalent to 10mg of Clobazam in a clean 10 ml volumetric flask and made up to the mark with distilled water.

Preparation of reagents: 0.25% $FeCl_3$ solution was prepared by dissolving 250mg of ferric chloride in double distilled water and made up to 100ml. 0.2% 1,10 phenonthroline and 0.1% potassium ferricyanide solutions were prepared by dissolving 200mg or 100mg of 1,10 phenonthroline or potassium ferricyanide in water and made up to 100ml. About 8.7ml of concentrated HCl was transferred into a clean 100ml volumetric flask and diluted up to the mark with distillated and the final concentration of the resulting solution was 1N HCl.

Method Development

Method-A (RP-HPLC): For developing RP-HPLC method, a systematic study of the effect of various factors such as detection wavelength, mobile phase and flow rate was undertaken by varying one parameter at a time and keeping all other conditions constant. After completion of different trails, the optimized chromatographic conditions were found to be Zodiac, C18 (250mmx4.6mm, 5µ particle size) as column, mixture of THF: MeOH: CAN in the ratio 10: 30: 60 (v/v) and pH was adjusted to 6.5 with 0.1%TEA as mobile phase at a flow rate of 0.8ml/min with a run time of 12 minutes and detection wavelength at 227 nm. Typical RP-HPLC chromatograms of Clobazam standard (R_t=6.145min) and sample (R_t=6.110min) were recorded by injecting 20µl of standard or sample and represented in Figure-2 & Figure-3 respectively. The system suitable parameters were presented in Table-1.

Detection wavelength: The UV absorption spectrum of diluted standard solution of the Clobazam was recorded by scanning in UV region (i.e.200-400nm) on UV spectrophotometer in spectrum mode. The absorption spectrum showed that the drug has

maximum absorbance at 227nm therefore the chromatographic analysis was carried out by choosing the 227nm as detection wavelength in UV detector of the HPLC system.

Choice of stationary phase: Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the desired separation and shapes of peak was achieved with analytical column Zodiac C-18 column of dimensions 250mm length, 4.6mm internal diameter and 5µm particle size.

Selection of the mobile phase: Based upon the solubility of the drug, initial developmental trails were performed on pure soluble solvents. It was found that acetonitrile give comparatively best results than water and methanol. Broad peaks and low theoretical plates were observed when acetonitrile was used as major solvent in combination with water and methanol as minor solvents. By changing different solvents, finally tetra hydro furan, methanol and acetonitrile in the ratio of 10:30:60 (v/v/v) was found to be suitable mobile phase. Sharp peak was observed with high theoretical plates and less tailing factor under this combination of solvents. The pH of optimized mobile phase was adjusted to be 6.5 with tri ethyl amine.

Selection of the mobile phase flow rate: Flow rate of the mobile phase was changed from 0.8 – 1.2 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 0.8ml/min flow rate was ideal for the successful elution of the analyte.

Method-B and Method-C (Visible spectrophotometric methods): In developing visible spectrophotometric methods, optimized conditions were developed by making different trails by varying one of the parameters such as concentration of standard, volume of the reagents, order of addition of the reagents, temperature and time for color development and keeping other as constant, and the optimized procedures were presented below.

Method-B : About 2.5ml of stock solution (300 µg/ml) was accurately transferred into 10 ml graduated test tube, about 0.5 ml of 0.25% $FeCl_3$ solution and 2.0 ml of 0.2% 1,10 phenonthroline were added. The tube was heated in water bath up to 30 min. after cooling to room temperature, about 2.0 ml of 1N HCl was added and made to 10 ml with distilled water. The absorbance of the colored solution was scanned over a range of wavelength 400-800nm against a reagent blank and the wavelength of maximum absorbance was found to be 542nm. The absorption spectrum was presented in Figure-4.This method was based on the formation of ferrous tris-o-phenanthroline complex with 1, 10 - phenanthroline at pH ~ 3±0.2.

Method-C: In this method, in a 10ml graduated test tube about 3.2ml of standard drug (100 µg/ml) and 1.0 ml of 0.25% $FeCl_3$ solution were taken and the tube was stoppard immediately and shaken well for 5 min. Then 0.5ml of 0.1% potassium ferricyanide solution was added and closed with lid immediately. After 5 min, 1.0ml of 1N HCl was added and the final volume was made up to 10 ml with distil water. The absorption spectrum was scanned from 400-800nm against a reagent blank and 698nm was found to be wavelength of maximum absorbance. The absorption spectrum was given in Figure-5. Green-yellow colored solution of potassium ferricyanide turns blue (prussian blue $Fe_4[Fe(CN)_6]_3$) in the presence of Fe^{2+} ions. Prussian blue, the deep blue pigment in blue printing, is generated by the reaction of $K_3[Fe(CN)_6]$ with ferrous (Fe^{2+}) ions.

Method Validation

Specificity: To find out specificity, about 20µL of blank (mobile phase), working standard and sample solutions were injected separately into the HPLC system and chromatograms were recorded under the optimized chromatographic conditions. In case of spectrophotometric analysis, absorbance was measured for the colored products of the standard and sample in Method-B and Method-C. The experimental results were given in Table-2.

Precision: The precision of the proposed methods was expressed in terms of intra-day and inter-day precision. The intraday and inter day precision were determined by measuring the response of the instrument (peak area in Method-A and absorbance in Method-B &C) six times within two different days by freshly preparing working standard solution and measuring the signal of the instrument following the standard procedures for the Method A, B and C. The precision was expressed either in terms of standard deviation or relative standard deviation (RSD), more over the better way of expressing the precision is percent of standard deviation (%RSD). The results of intraday and inter day precision of the proposed method were presented in Table-3 and Table-4 respectively.

Accuracy: Accuracy of the proposed methods was determined by calculating percent of recovery of Clobazam by the method of standard addition. A

known amount of Clobazam standard was added to a pre analyzed sample and the amount of Clobazam was estimated by measuring the response of the instrument (peak area in Method-A and absorbance in Method-B &C) three times over the specified concentration range (80%, 100% and 120% with respect to target concentration) and amount of Clobazam was estimated by following the standard procedures for three methods. The accuracy results were given in Table-5.

Linearity: Appropriate aliquots of standard Clobazam stock solution (1.0-9.0 of 1000µg/ml for Method-A) were taken in a series of volumetric flasks and made up to the mark. The response of the instrument (peak area) was measured twice at each concentration, and calibration curve was constructed by plotting peak area versus concentration of Clobazam. The linearity plot was presented in Figure-6. In Method-B, different aliquots of stock solution (1.0-5.0ml of 300 µg/ml) was accurately transferred into a series of 10 ml graduated test tubes, about 0.5 ml of 0.25% $FeCl_3$ solution and 2.0 ml of 0.2% 1,10 phenonthroline were added. The tubes were heated in water bath up to 30 min. after cooling to room temperature, about 2.0 ml of 1N HCl was added to each tube and made to 10 ml with distilled water. The absorbance of the colored solution was measured after 5min at 542nm against a reagent blank and a linear plot was drawn absorbance against concentration and represented in Figure-7. In case of Method-C, into a series 10ml of calibrated tubes, aliquots of standard drug (1.6 -5.6 ml of 100 µg/ml) are transferred and 1.0 ml of 0.25% $FeCl_3$ solution was added. The tubes were stoppard immediately and shaken well for 5 min. Then 0.5ml of 0.1% potassium ferricyanide solution was added into each tube and was closed with lids immediately. After 5 min, 1.0ml of 1N HCl was added and the final volume was made up to 10 ml with distil water. The absorbance was measured at 698nm against a reagent blank and calibration plot was constructed and presented in Figure-8. The results of linearity in these methods were presented in Table-6.

Sensitivity: The sensitivity of the proposed method was presented in terms of limit of detection (L.O.D.) and limit of quantification (L.O.Q.) and these were calculated from standard deviation of response and slope of the calibration curve(s) by using the formulae LOD = $3S_a$ / b and LOQ = $10S_a$ / b respectively and given in Table-7.

Formulation analysis: The prepared sample solution was analyzed by adopting the standard procedures and percent of assay was determined, and the assay results were given in Table-8.

Ruggedness: The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc. In the present investigation, robustness was tested by ±5% change in the mobile phase ratio, wavelength of the detector and the pH of the detector at a concentration of 50µg/ml solution. It was found that there is no remarkable change in the results with all the changed conditions.

Robustness: Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. Parameters that should be investigated are percent organic content in the mobile phase, pH of the mobile phase, buffer concentration, and temperature and injection volume. Ruggedness was tested by changing the operator in two different days at 50µg/ml concentrations and %RSD was calculated and was found to be under the acceptance criteria.

RESULTS AND DISCUSSION

The main objective of the present investigation was to develop and validate a liquid chromatographic and two visible spectrophotometric methods using 1, 10 phenanthroline and ferricyanide as complexing agents for color development for the determination of Clobazam in pure and formulations. The system suitable parameters such as peak area, theoretical plates, tailing factor and retention time of the standard and sample were found to be 852229.4&852348.9, 13107&12984, 0.870&0.864 and 6.145&6.110min respectively. Mean, standard deviation, relative standard deviation and percent of relative standard deviation in the study of intraday precision were found to be 854954.5 (0.535&0.450), 2533.75 (0.0021&0.0022), 0.0029 (0.0040&0.0048) and 0.2963 (0.4035& 0.4793) respectively for the three methods. Similarly the inter day precision of these methods was found to within the limits. The %RSD in intraday and inter day precision studies was found to be less than 2.0% indicate that the proposed methods were precise. The accuracy of the methods was determined as percent of recovery, and performed at three different concentrations and found to be in the range of 99.84-101.74%, this indicate that the developed methods were accurate. The studies of linearity between the response and concentration of the drug indicate that these methods were linear within the best suitable rage (15-90, 30-105 and 16-56µg/ml for Method-A, B and C respectively) and

correlation coefficient was found to be not less than 0.9990. The LOD and LOQ values of the proposed methods were determines and found to be 0.051 (2.2 &1.2) and 0.150 (7.3 & 4.0). The study of ruggedness and robustness was carried out and results proved the developed Method-A was rugged and robust. Assay of formulations was determined and the results were satisfactory, hence these methods may adopt in any quality control laboratory.

CONCLUSIONS

The proposed methods were found to be simple, sensitive, precise, accurate, and linear over a suitable range, robust and rugged. The tablet dosage forms were analyzed successfully and the assay was found to be within the limits, hence the proposed methods may be applied for the quality analysis.

ACKNOWLEDGMENTS

The authors are grateful to R.V Labs Guntur for providing laboratory facilities and university authorities, Acharya Nagarjuna University, Guntur for getting registration.

Table-1: System suitable parameters for Clobazam

S.No.	Parameter	Standard	Sample
1	Retention time	6.145min	6.110 min
2	Peak area	852229.4	852348.9
3	Theoretical plates	13107	12984
4	Tailing factor	0.870	0.864
5	Peak height	112471	112418

Table-2: Specificity of the proposed methods

	Method-A	Method-B	Method-C
Solution	**Area**	**Absorbance**	**Absorbance**
Standard	852229.4	0.539	0.453
Sample	852348.9	0.541	0.457
Blank	---	---	---

Table-3: Intraday Precision results for Clobazam

S.No.	Method-A Concentration µg/ml	Peak Area	Method-B Concentration µg/ml	Absorbance	Method-C Concentration µg/ml	Absorbance
1	50	855879	75	0.537	32	0.451
2	50	850602	75	0.535	32	0.454
3	50	858421	75	0.536	32	0.45
4	50	855241	75	0.538	32	0.452
5	50	855065	75	0.534	32	0.449
6	50	854519	75	0.532	32	0.448
	Mean	854954.5		0.535		0.450
	SD	2533.75		0.0021		0.0022
	RSD	0.0029		0.0040		0.0048
	CV	0.2963		0.4035		0.4793

Table-4: Interday Precision results for Clobazam

S.No.	Method-A Concentration µg/ml	Peak Area	Method-B Concentration µg/ml	Absorbance	Method-C Concentration µg/ml	Absorbance
1	50	853204	75	0.518	32	0.435
2	50	869708	75	0.514	32	0.437
3	50	846755	75	0.516	32	0.436
4	50	861646	75	0.517	32	0.434
5	50	850844	75	0.515	32	0.438
6	50	856388	75	0.513	32	0.433
	Mean	856424.17		0.515		0.435
	SD	8230.79		0.0018		0.0018
	RSD	0.0096		0.0036		0.0043
	CV	0.9610		0.3629		0.4296

Table-5: Accuracy results for the proposed methods

Method	Spike level	Target Concentration (µg/ml)	Spiked Concentration (µg/ml)	Total Concentration (µg/ml)	Concentration Obtained* (µg/ml)	% Recovery*
Method-A	50%	30	15	45	45.01	100.02
	100%	30	30	60	60.02	100.02
	150%	30	45	75	74.85	99.84
Method-B	50%	30	15	45	45.63	101.46
	100%	30	30	60	60.31	100.5
	150%	30	45	75	75.36	100.46
Method-C	50%	16	8	24	24.27	100.14
	100%	16	16	32	32.23	101.73
	150%	16	24	40	40.25	100.64

**** Average of three determinations***

Table-6: Linearity between signal and concentration of Clobazam

	Method-A		Method-B		Method-C	
S.No.	Concentration in µg/ml	Peak Area	Concentration in µg/ml	Absorbance	Concentration in µg/ml	Absorbance
1	15	311047	30	0.231	16	0.231
2	30	588228	45	0.342	24	0.346
3	45	852229	60	0.431	32	0.453
4	60	1092789	75	0.539	40	0.561
5	75	1389708	90	0.649	48	0.653
6	90	1649399	105	0.762	56	0.786
	Slope	18119		0.007		0.013
	Intercept	25120		0.008		0.006
	Correlation coefficient	0.9995		0.9996		0.9994

Table-7: Sensitivity of the proposed method expressed as LOD and LOQ

S.No.	LOD	LOQ
Method-A	0.051	0.150
Method-B	2.2	7.3
Method-C	1.2	4.0

Table-8: Formulation Analysis results of Clobazam

S.No.	Brand name	Dosage form	Label claimed mg	Amount obtained	% Assay
Method-A	Frisium	Tablet	20	19.92	99.6
Method-B	Frisium	Tablet	20	19.96	99.8
Method-C	Frisium	Tablet	20	19.90	99.5

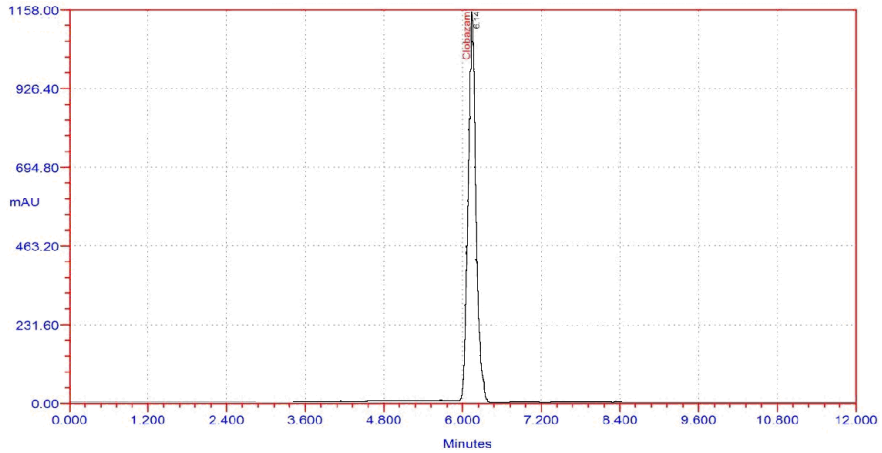

Figure-2: RP-HPLC chromatogram of Clobazam standard (R_t=6.145minutes)

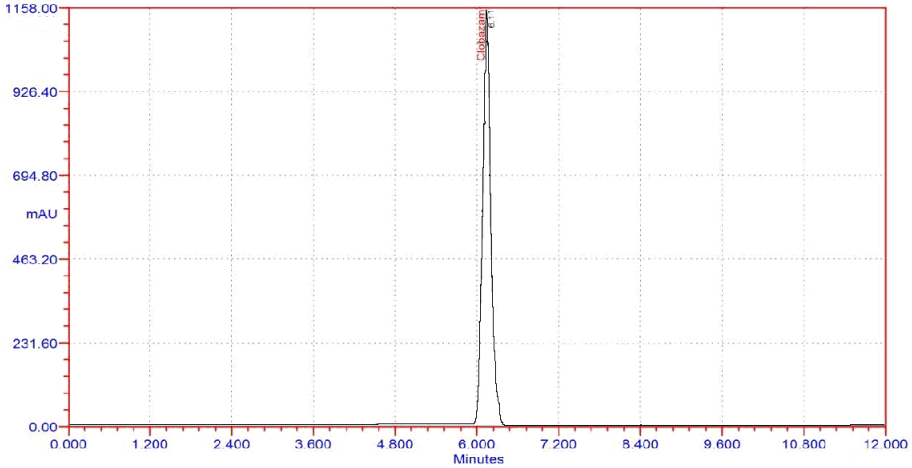

Figure-3: RP-HPLC chromatogram of Clobazam sample (R_t=6.110minutes)

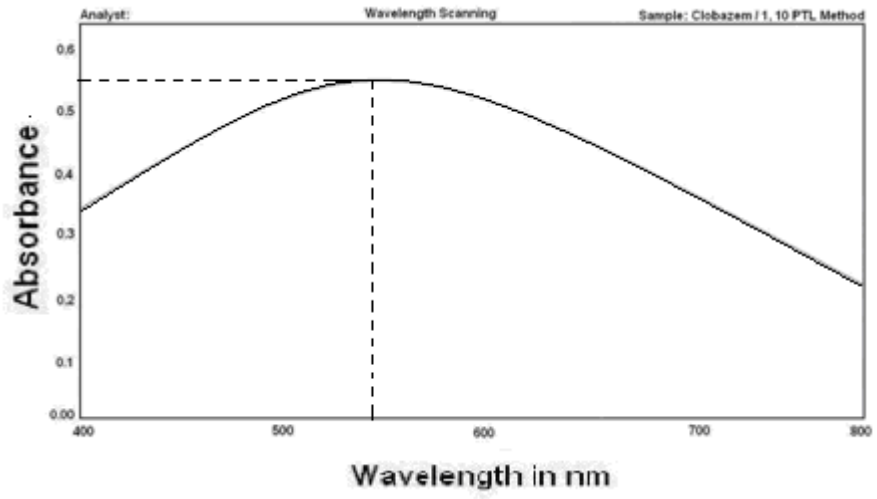

Figure-4: Absorption spectrum of colored product in Method-B

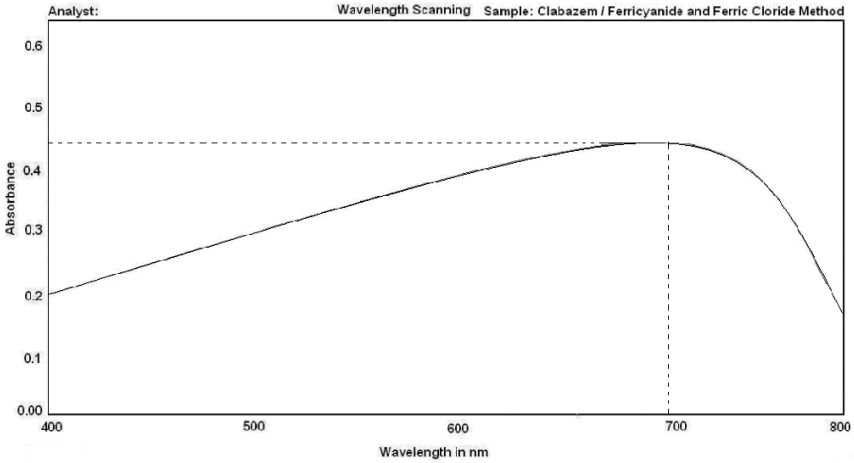

Figure-5: Absorption spectrum of colored product in Method-C

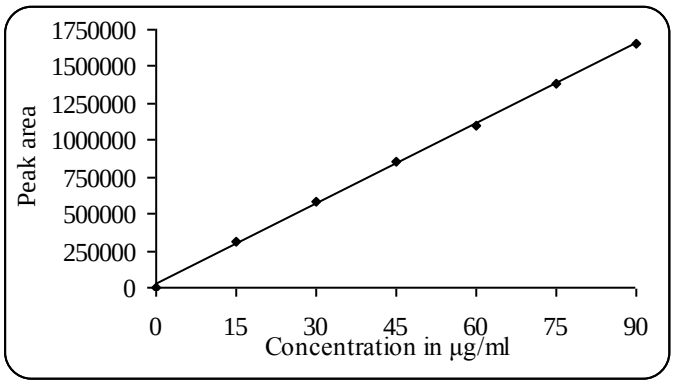

Figure-6: Linearity between area and concentration of drug (Method-A)

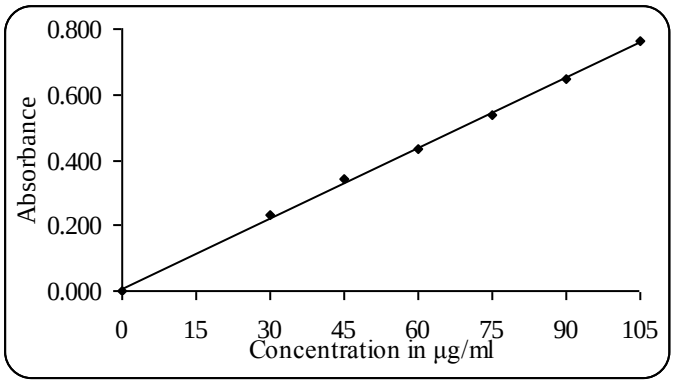

Figure-7: Linearity between absorbance and concentration of drug (Method-B)

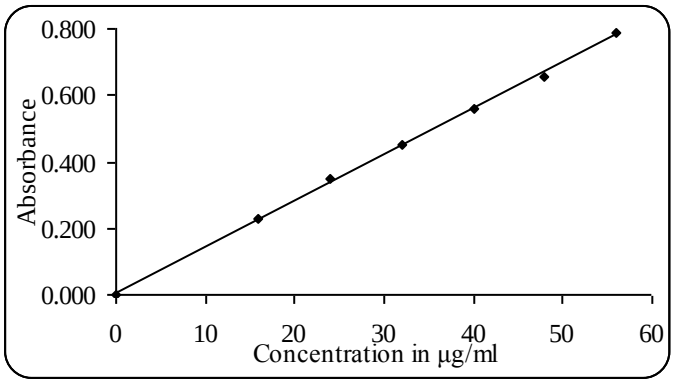

Figure-8: Linearity between absorbance and concentration of drug (Method-C)

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