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Research Article

RP-HPLC TECHNIQUE DEVELOPMENT AND VALIDATION TO ASSESS CLOBAZAM STABILITY

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ABSTRACT
Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. This analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as chromatography. UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.

Keywords: Quality control, Standards of Medicine, Clobazam, RP-HPLC

INTRODUCTION

Quality can be defined as the character, which defines the grade of excellence. A good quality drug is something, which will meet the established product specifications, can be safely bought and confidently used for the purpose for which it is intended. To get a good quality drug, the manufacturing for making a drug should have quality built into it [1].

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials [2]. Analytical chemistry is a sub discipline of chemistry that has the broad mission of understanding the chemical composition of all matter and developing the tools to elucidate such compositions [3].

The analytical way deals with quality standards which are assigned for products to have desirable efficacy of the medicines [4]. Sample representing any batch are analyzed for these standards and it is assumed that drug/medicine which is having such standards are having desire effect on use. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action [5].

Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. This analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as 'CHROMATOGRAPHY' [7].

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METHODOLOGY

UV-Spectral Analysis of Clobazam

Instrumentation

Instruments used were UV-visible double beam spectrophotometer model Shimadzu UV1800 with one cm matched quartz cells and AJ-Vibra electronic balance manufactured by Essae Teraoka Ltd., Made in Japan. The glass wares used in each procedure were soaked overnight in a mixture of chromic acid and sulphuric acid rinsed thoroughly with double distilled water and dried in hot air oven for prior use. The absorption spectra of standard were carried out in a one cm quartz cells over the range of 200-400 nm.

Preparation of standard stock solution

Weigh accurately 10 mg of Clobazam into 10 ml volumetric flasks. Then add sodium hydroxide and methanol in the ratio of 80:20 to dissolve the drug and then volume was made up to 10 ml with mobile phase. The concentration of standard stock solution is 1 mg/ml.

Preparation of working standard solution

Transferred 5 ml from the above standard stock solutions in to 50 ml volumetric flasks and diluted up to the mark with mobile phase to get working standard solution of concentration 0.1 mg/ml. From this 2ml is diluted to 10 ml to get 20µg/ml.

Determination of λ max

Most of drugs absorb light, UV wavelength (200-400 nm) since that contains aromatic double bonds. The solution containing 10µg/ ml of Clobazam was prepared and scanned over the range of 200-400 nm against acetonitrile as blank using Shimadzu UV1800 double beam UV spectrophotometer.

Calibration Curve for Clobazam

From the stock solution, a concentration of various dilutions gives 30, 40, 50, 60, 70, 80, 90 µg/ml concentration of clobazam respectively. The absorbance was measured using UV spectrophotometer [8].

Method development of Clobazam drug by RP-HPLC

Instrumentation

Instrument used – Shimadzu Model CBM-20 A/20 Alite HPLC system equipped with an SPD M20A prominence photodiode array detector (250 mm×4.6 mm, 5 mm particle size) maintained at 25^{o}C.

Elution type - Isocratic elution

Mobile Phase- acetonitrile and water (60:40, v/v)

Flow rate- 0.8ml/min

Sample volume- 20µL

Stock solution- 1000 µg/ml

Preparation of stock solutions

Clobazam stock solution (1000 µg/ml) was prepared by weighing 50 mg of clobazam in a 50 mL amber volumetric flask and making up to volume with mobile phase. Working solutions for HPLC injections were prepared on a daily basis from the stock solution in a solvent mixture of acetonitrile and water (60:40, v/v) (mobile phase). Solutions were filtered through a 0.45 mm membrane filter prior to injection.

25 tablets from each brand (CLOBA and CLOZAM) were procured, weighed and crushed to a fine powder. Powder equivalent to 50 mg Clozabam was accurately weighed into a 50 mL volumetric flask and made up to volume with mobile phase. The contents of the volumetric flask were sonicated for 30 min to enable complete dissolution of Clozabam. The solution was filtered and the filtrate was diluted with mobile phase. 20 mL of these solutions were injected into the system and the peak area was recorded from the respective chromatogram [9].

Method Validation

The method was validated for the following parameters: linearity, precision, accuracy, selectivity, robustness, limit of quantitation (LOQ), limit of detection (LOD) and system suitability [10].

System Suitability Test

System suitability test is an integral part of the chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use [11].

Linearity

The linearity of an analytical procedure is its ability (with in a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample.

Linearity of an analytical procedure is established minimum of five concentrations. It is established initially by visual examination of plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results are established by appropriate statistical methods (i.e., by calculation of the regression line by the method of least squares).

Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels and 20 µL of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted [12].

Precision

The precision of an analytical procedure express the closeness of the agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or co-efficient of variation of a series measurement.

System precision

A system precision is evaluated by measuring the peak response for the six replicable injection of the same standard solution prepared as per the proposed

method. The %RSD is calculated and it should not be more than 2%.

Method precision

A method precision is evaluated by measuring the peak response for six replicate injection of six different weigh of sample solution prepared as per proposed method. The %RSD is calculated and it should not be more than 2%.

Determination

The intra-day precision of the assay method was evaluated at three concentration levels (10, 20 and 50 mg/mL) (n=3) against a qualified reference standard. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (10, 20 and 50 mg/mL) (n=3). The %RSD of the obtained assay values at three different concentration levels was calculated.

Recovery Study (Accuracy)

The accuracy of the assay method was evaluated in triplicate at three concentration levels (80, 100 and 120%), and the percentage recoveries were calculated. The study was carried out in triplicate at 18, 20 and 22 µg/Ml

Limit Of Detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value.

ICH has recommended some method for determining the limit of detection. The method may be either instrumental or non-instrumental. Limit of detection (LOD) based on standard deviation of the response and the slope of calibration curve.

$$LOD = \frac{3.3\,s}{S}$$

S = Slope of calibration curve

S = Standard deviation of the response

Limit Of Quantification (LOQ)

The limit of Quantitation (LOQ) is defined as the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

Limit of Quantitation (LOQ) is also based on standard deviation of the response and the slope of calibration curve.

$$\mathbf{LOQ} = \frac{10\,s}{S}$$

S = Slope of calibration curve

S = Standard deviation of the response

Determination of the detection and quantification limits was performed based on the standard deviations of y-intercept and the slope of the least square line parameters as defined in the International Conference on Harmonization (ICH) Q2 guidelines

Robustness

The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength, percentage of acetonitirile in the mobile phase (58 and 62) and flow rate (0.7 and 0.9 mL/min). Robustness of the method was studied using six replicates at a concentration level of 20 µg/mL of Clobazam

Forced Degradation Studies/Specificity

Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. The study was intended to ensure the effective separation of clobazam and its degradation peaks of formulation ingredients at the retention time of clobazam.

All solutions for use in stress studies were prepared at an initial concentration of 1 mg/mL of clobazam and refluxed for 30 min at 80^{o}C. All samples were then diluted in mobile phase to give a final concentration of 50 mg/mL and filtered before injection.

Acid And Alkaline Hydrolysis

Acid decomposition was carried out in 0.1M HCl and alkaline degradation was conducted using 0.1 M NaOH and refluxed for 30 min at 80^{o}C. After cooling the solutions were neutralized and diluted with mobile phase.

Oxidative Degradation

Solutions for oxidative stress studies were prepared using 3% H_2O_2 at a concentration of 1 mg/mL of Clobazam and after refluxation for 30 min at 80^{o}C on the thermostat the sample solution was cooled and diluted accordingly with the mobile phase.

Thermal Degradation

For thermal stress testing, the drug solution (1 mg/mL) was heated in thermostat at 80 1C for 30 min, cooled and used. The drug solution (1 mg/mL) for photo stability testing was exposed to UV light for 4 h UV light chamber (365 nm) and analysed [13].

Results And Discussion

UV-Spectral Analysis of Clobazam

With the help of a UV spectrophotometer and with dilutions of medication (20µg/ml) in solvent the absorbance of bivalirudin in the UV range of 200-400 was determined. [14,20] At 230 nm, the maximum

absorbance was determined and thus the absorption maximum of the drug was determined. The results were shown in figure 3.[21,24]

Preparation of calibration curve

Concentration of various dilutions 30, 40, 50, 60, 70, 80, 90 µg/ml concentration of clobazam has done. The regression values were also calculated to be 0.997, and the calibration values have been shown in table 1.

Chromatogram of Clobazam

Clobazam stock solution (1000 µg/ml) was prepared by weighing 50 mg of clobazam in a 50 mL amber volumetric flask and making up to volume with mobile phase. [25,28] Working solutions for HPLC injections were prepared on a daily basis from the stock solution in a solvent mixture of acetonitrile and water (60:40, v/v) (mobile phase). Solutions were filtered through a 0.45 mm membrane filter prior to injection.

20 µL of these solutions were injected into the system and the peak area was recorded from the respective chromatogram.[29]

The representative chromatogram obtained for Clobazam is shown in Fig. 5 A and those of marketed formulations are shown in Fig.5 B–C.[30]

Method Validation

The method was validated as per ICH guidelines with respect to parameters defining linearity, precision, accuracy, LOD&LOQ, Repeatability and Recovery study.

The number of theoretical plates, peak tailing and resolution factor were determined to define system suitability parameters for Clobazam. The results for system suitability data are listed in Table 2

Validation Parameters

Linearity and Range

The linearity of response obtained between 30 to 90 µg/ml concentrations and calibration curve were obtained by plotting absorbance versus concentration data and treated by linear regression analysis.[32,37] The calibration curve equation for clobazam is Y= 0.0248x and calibration curve was found to be linear in the above mentioned concentration and correlation coefficient (R^2) was 0.9977. The linearity and range resulted from regression analysis of bivalirudin was found to be 30-90µg/ml.

Precision

Repeatability

Repeatability has been determined by analyzing samples 60 µg/ ml of clobazam for six times and measured at 230nm. The results are reported in Table 3

Inter-day precision

This was done by analyzing formulation by same analyst but for six days subsequently. The results are summarized in table 4

Figure 1: Schematic diagram of HPLC

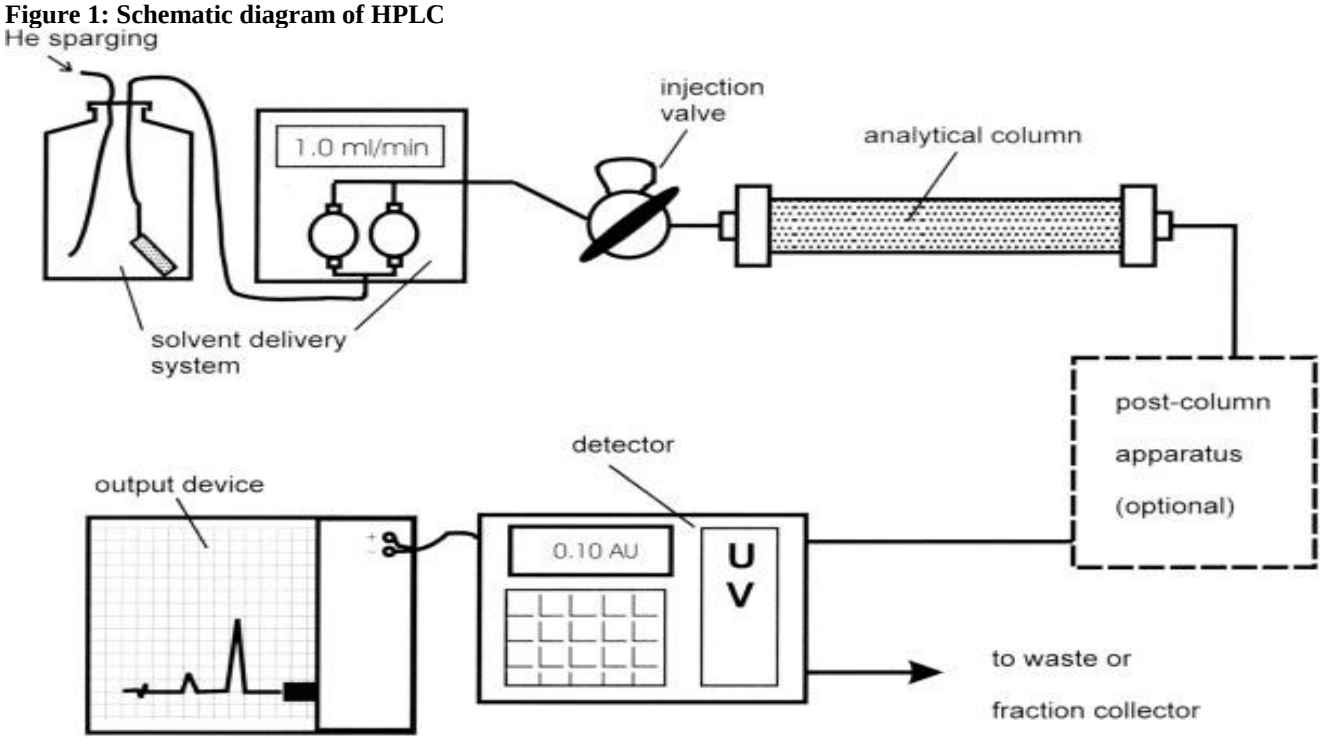

Figure 2: Method validation Parameters

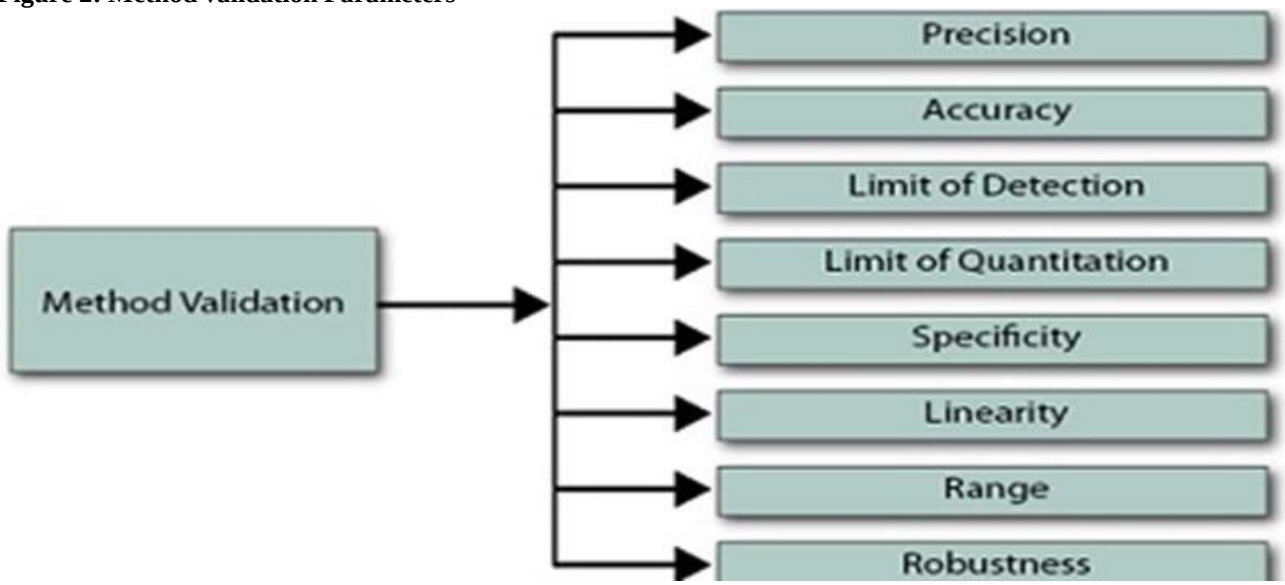

Figure 3: Absorption spectra of clobazam

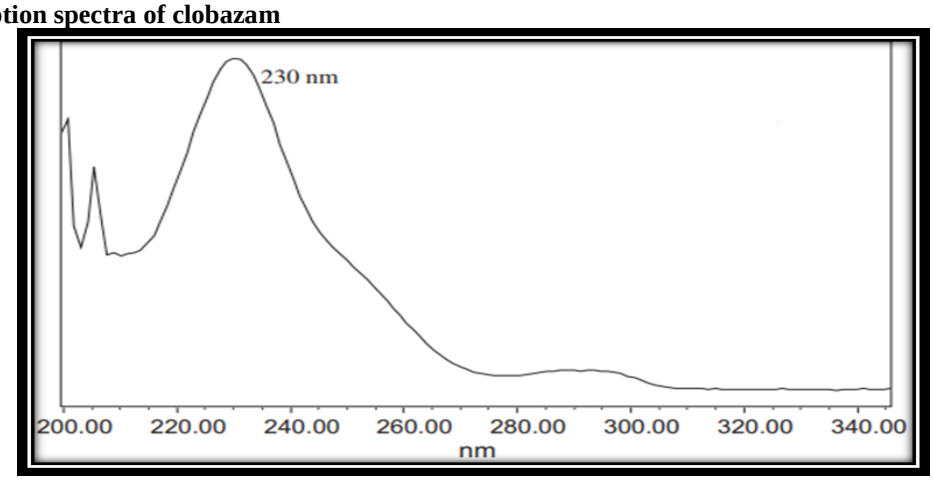

Figure 4: Linearity curve of Clobazam

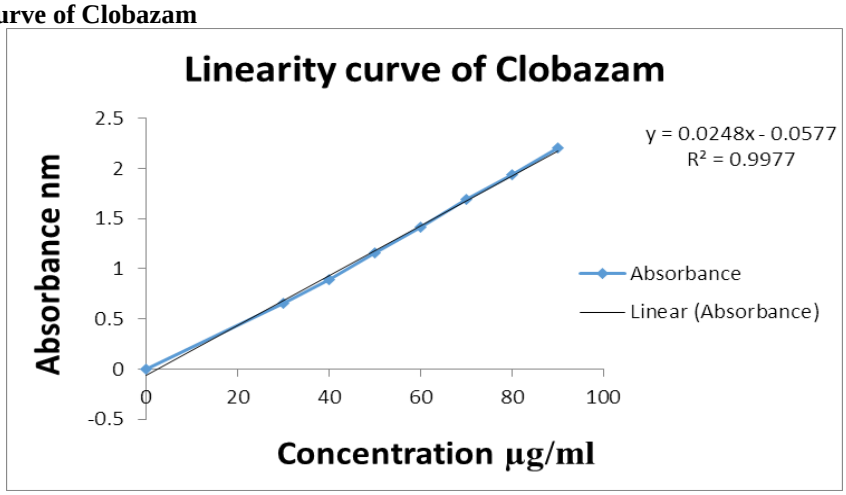

Figure 5: Representative chromatograms of Clobazam (50 mg/mL) (A), Cloba (400 mg) (B), and Clozam (400 mg) (C)

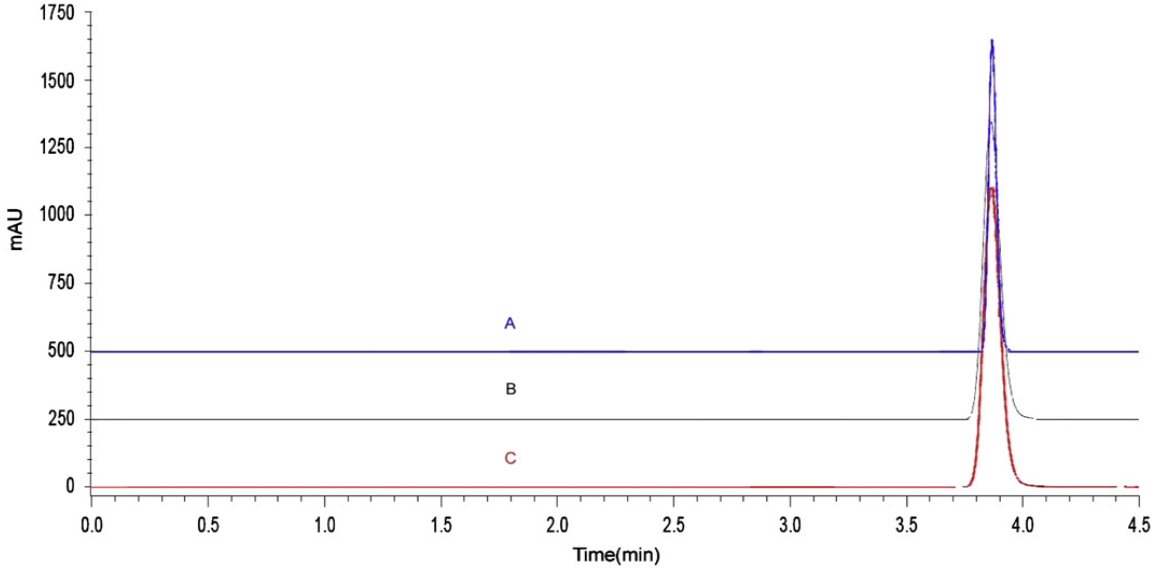

Figure 6: Representative chromatogram of clobazam (50µg/mL), on Acidic (A), alkaline (B), Oxidative (c), Photolytic (D), and Thermal (E) degrtadation

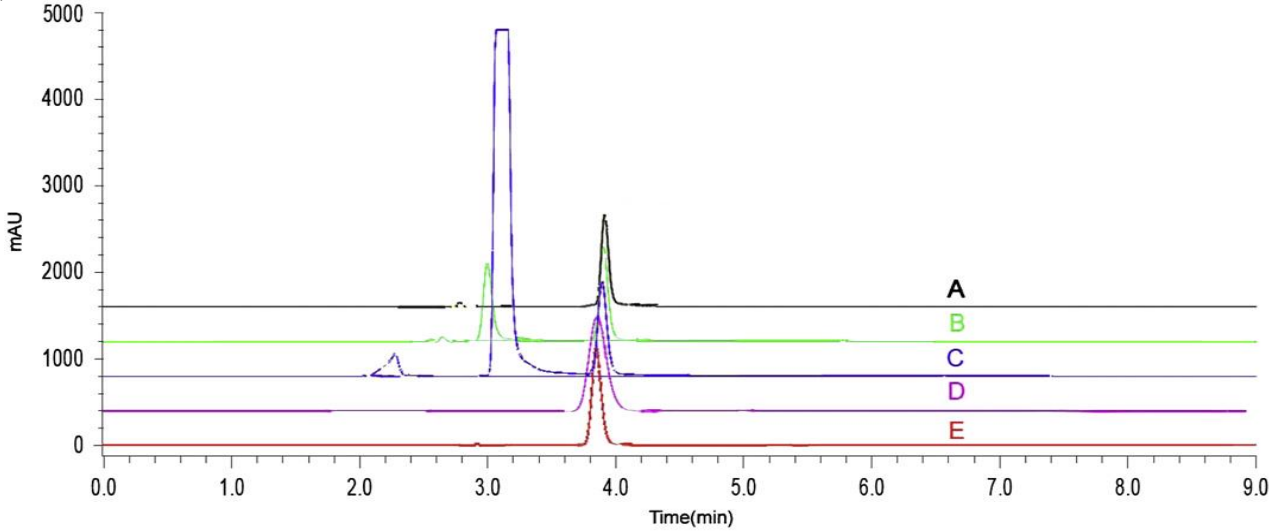

Table 1: Calibration data of Clobazam

S. No	Concentration (µg/ml)	Absorbance (nm)
1	30	0.651
2	40	0.891
3	50	1.158
4	60	1.412
5	70	1.695
6	80	1.941
7	90	2.201

Table 2: System suitability parameters

S.NO	System Suitability Parameters	Clobazam
1	Theoretical plates per column	8576
2	Symmetry factor/tailing factor	1.27

3	Retention Time	3.891

Table 3: Intraday precision of clobazam

S.No	Concentration (µg/ml)	Absorbance at 230 nm	Statistical analysis (n=6)
1	60 µg/ml	1.412	Mean=1.416±0.006
2		1.405	
3		1.419	
4		1.423	
5		1.418	%RSD= 0.42
6		1.420	

Table 4: Interday precision of clobazam

S.No	Concentration (µg/ml)	Day	Absorbance at 230 nm	Statistical analysis at 255 (n=6)
1	60 µg/ml	1	1.411	Mean=1.407±0.008
2		2	1.399	
3		3	1.405	
4		4	1.411	
5		5	1.419	%RSD= 0.56
6		6	1.398	

Table 5: Accuracy Data of Clobazam

S. No	Recovery	Concentration	Absorbance	Amount found	% Recovery
1	25%	50ppm	1.141	49.90	99.81
		50ppm	1.143	49.91	99.63
		50ppm	1.140	48.55	99.9
2	50%	60ppm	1.396	59.88	99.78
		60ppm	1.397	59.96	99.87
		60ppm	1.396	59.96	99.89
3	100%	80ppm	1.903	79.95	99.89
		80ppm	1.90	79.81	99.81
		80ppm	1.901	79.85	99.91

Table 6: Percentage Assay of Clobazam

Brand Name	Label claim	Concentration	Absorbance	Average	%Assay
Onfi	10 mg	60ppm	1.415	1.413	99.97
Onfi	10 mg	60ppm	1.411		
Onfi	10 mg	60ppm	1.413		

Table 7: Forced degradation studies of Clobazam

Stress condition	Drug recovered (%)	Drug decomposed (%)
Standard drug	100	0
Acidic hydrolysis	92.20	7.80
Alkaline Hydrolysis	97.15	2.85
Oxidative degradation	94.35	5.65
Thermal degradation	99.94	0.06
Photolytic degradation	99.85	0.15

ACCURACY

The accuracy, specificity, suitability and validity of the proposed methods were satisfied by conducting recovery studies.[38] A known quantity of the drug was added to the pre analyzed sample formulation at 25%, 50% and 100% levels. The percentage recovery was calculated and given in table 5

Limit of detection & limit of quantification

The Limit of Detection **(**LOD) and Limit of Quantification **(**LOQ) were 0.2158 µg/ ml and 0.6915 µg/ml respectively.

Estimating the commercially available clobazam dosage form

Twenty tablets were weighed accurately and the average weight of each tablet was calculated. The tablets

were powdered well with the help of glass mortar and pestle. Tablet powder equivalent to 1 tablet weight was weighed accurately and transferred to a 10 ml volumetric flask.[39] Then add small quantity of methanol: 1 NaOH and sonicate it for 30 min to dissolve the drugs completely and then the volume was made up to the mark with the diluent and filtered through 0.45 µm membrane filters.[40] From this, 2 ml was taken and diluted to100 ml with diluents to get 20 µg/ml concentrations. The absorbance of this solution was measured at 230 nm against diluent as a blank. This procedure was repeated 3 times. The results are summarized in table 6

Establishment of stability indicating method for assessment of degradation behaviour

The stressed samples were assayed using developed RP-HPLC method. The following degradation behavior was observed under different stress conditions for the high-performance liquid chromatography studies on clobazam. [41]

The result of various stability assessments on clobazam given in table 7. Typical chromatograms obtained following the assay of stressed samples are shown in fig 6.

CONCLUSION

In order to develop an effective RP-HPLC method, most of the effect should be spent in method development and optimization as this will improve the final method performance. A well-developed method should be easy to validate. Keeping in this mind we developed methods for determination and validation of clobazam in bulk and pharmaceutical dosage forms by RP- HPLC with some improvements than the existing methods.

The analytical procedure described for assay was specific, linear, precise, accurate, and system suitable for determination of Clobazam in bulk and pharmaceutical dosage forms. The observations of the validation parameters such as accuracy, precision, specificity, linearity, shows that the developed methods can be employed for routine analysis of bulk and tablets form of clobazam. The result obtained from the validation parameters met the ICH and USP requirement as well as obeys BEER'S law.

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