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RPHPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOBAZAM AND FORCED DEGRADATION STUDIES

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ABSTRACT

The spectrophotometric method developed for quantifying Clobazam in bulk and finished pharmaceutical preparations is highly accurate, sensitive, rapid, precise, and economical. Utilizing UV spectrophotometric absorption at 230 nm, the method demonstrates a low Limit of Detection (LOD) of 0.2158 µg/ml and a Limit of Quantification (LOQ) of 0.6915 µg/ml, showcasing its ability to detect and quantify low concentrations of Clobazam. A strong linear relationship was observed between 30-90 µg/ml with a regression equation of Y = 0.0248x and a correlation coefficient of 0.997, indicating excellent linearity. Accuracy was validated with mean percentage recovery ranging from 99.50% to 100%. Precision studies revealed a Relative Standard Deviation (RSD) of 0.42% for intra-day and 0.56% for inter-day measurements, ensuring consistent results. The method's robustness was confirmed with RSD values below 2.0% under varied conditions. Application to Clobazam tablets yielded assay results of 99.97% with RSD values below 2.0%, demonstrating suitability for routine analysis. Chromatographic parameters showed a capacity factor greater than 2, theoretical plates numbering 8576, and a tailing factor of 1.27, indicating excellent performance. In degradation studies, Clobazam showed less than 6.0% decomposition under thermal, oxidative, and UV conditions. Acidic degradation resulted in 7.80% decomposition, and alkaline conditions produced a major degradant at 2.987 minutes without interfering with the main drug peak at 3.891 minutes, with a drug decomposition percentage of 2.85%. This method reliably distinguishes between Clobazam and its degradation products, making it suitable for precise and accurate analysis even under stressed conditions.

KEY WORDS: Clobazam Quantification, UV Spectrophotometry, Analytical Method Validation, Pharmaceutical Analysis, Sensitivity and Precision, Degradation Studies

INTRODUCTION

The rapid growth of the pharmaceutical industry in recent years has led to the introduction of numerous pharmaceutical formulations into the healthcare system [1]. Consequently, there has been significant progress in the field of pharmaceutical analysis. Developing analytical methods for newly introduced pharmaceutical formulations is of paramount importance [2], especially when the drug or drug combination is not yet official in any pharmacopoeias, and no established analytical methods for quantification exist [3]. High-Performance Liquid Chromatography (HPLC) has become a cornerstone in pharmaceutical analysis, providing improved separation, identification, purification, and quantification capabilities far surpassing

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previous chromatographic techniques [4]. By the 1980s, HPLC was commonly used for the separation of chemical compounds, addressing the inadequacies of earlier chromatographic methods in quantifying compounds and resolving similar substances [5]. The reliability, speed, and sophistication of liquid chromatographic (LC) separation techniques have become essential in various industries, including pharmaceuticals, agrochemicals, dyes, petrochemicals, and natural products [6]. HPLC employs two basic elution modes: isocratic elution and gradient elution. In isocratic elution, the mobile phase composition remains constant throughout the run, while in gradient elution, the mobile phase composition changes during the separation [7]. Gradient elution is particularly useful for samples containing compounds with a wide range of chromatographic polarities [8]. A higher sampling rate is necessary in HPLC data acquisition systems to accommodate the rapidly eluting narrow peaks typical of HPLC chromatograms. Most drugs in single or multi-component dosage forms can be analyzed by HPLC due to its advantages, including rapidity, specificity, accuracy, precision, and ease of automation [9]. HPLC offers various modes of separation: normal phase mode, reversed-phase mode, reverse phase ion pair chromatography, affinity chromatography, and size exclusion chromatography (gel permeation and gel filtration chromatography) [10]. Reversed-phase mode is the most popular for analytical and preparative separations of compounds in chemical, biological, pharmaceutical, food, and biomedical sciences. Given that most drugs and pharmaceuticals are polar, they elute faster and are not retained for long periods [11]. Commonly used columns in reversed-phase HPLC include Octa Decyl Silane (ODS) or C18, C8, and C4, listed in increasing polarity of the stationary phase [12]. Clobazam, a long-acting antiepileptic drug from the benzodiazepine class, is used in the polytherapy of childhood epilepsy. Marketed under brand names such as Frisium and Onfi, Clobazam was patented in 1968 and first synthesized in 1966. Initially marketed as an anxioselective anxiolytic in 1970 and as an anticonvulsant since 1984, Clobazam aims to provide greater anxiolytic and antiobsessive efficacy with fewer benzodiazepine-related side effects. It is approved as adjunctive treatment for seizures associated with Lennox-Gastaut syndrome in patients aged two years or older. Due to its CNS depressant effects, patients or their caregivers should be cautioned about the concurrent use of other CNS depressants or alcohol, as Clobazam can potentiate their effects. Clobazam exerts its effects by binding to distinct sites on the post-synaptic GABA receptor-associated chloride ionophore [13], enhancing the inhibitory effect of GABA through increased chloride ionopore opening duration, resulting in membrane hyperpolarization and stabilization. The aim of the current study is to develop and validate an RP-HPLC method for the stability assessment of Clobazam, ensuring its efficacy and safety in pharmaceutical formulations.

METHODOLOGY

Reagents and chemicals

Clobazam reference standard was provided by centaur pharmaceuticals (Mumbai), formulation tablets were purchased from a local pharmacy. Acetonitrile (HPLC Grade), Sodium hydroxide, Hydrochloric acid and hydrogen peroxide were purchased from local supplier. Clobazam was available as tablets with brand names CLOBA and CLOZAM. All the chemicals were of analytical grade.

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. [14]

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 [15].

Method development of Clobazam drug by RP-HPLC [16]

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.

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.

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 80oC. All samples were then diluted in mobile phase to give a final concentration of 50 mg/mL and filtered before injection. [17]

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^{o}C 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 analyzed.

RESULTS

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. At 230 nm, the maximum absorbance was determined and thus the absorption maximum of the drug was determined. The results were shown in figure 1.

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 figure 2.

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. 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.

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 1

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. 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 (R2) 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 2.

Inter-day precision:

This was done by analyzing formulation by same analyst but for six days subsequently. The results are summarized in table 3

Accuracy

The accuracy, specificity, suitability and validity of the proposed methods were satisfied by conducting recovery studies. 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 4.

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.

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. The result of various stability assessments on clobazam given in table 5. Typical chromatograms obtained following the assay of stressed samples are shown in fig 3.

Table: 1 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: 2 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: 3 Interday precision of clobazam

S.No	Concentration (µg/ml)	Day	Absorbance at 230 nm	Statistical analysis (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: 4 Accuracy Data of Clobazam

S.No	Recovery	Concentration	Absorbance	Amount found	% Recovery
1	20%	50ppm	1.141	49.90	99.81
			1.143	49.91	99.63
			1.140	48.55	99.9
2	50%	60ppm	1.396	59.88	99.78
			1.397	59.96	99.87
			1.396	59.96	99.89
3	100%	80ppm	1.903	79.95	99.89
			1.90	79.81	99.81
			1.901	79.85	99.91

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

Figure: 1 Absorption spectra of clobazam

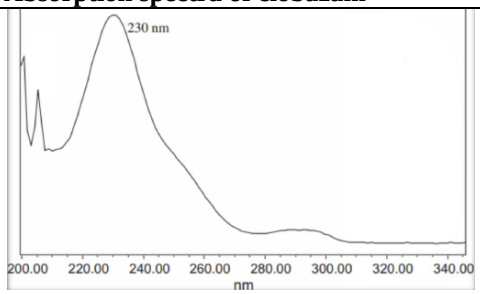


Figure: 2 Linearity curve of Clobazam

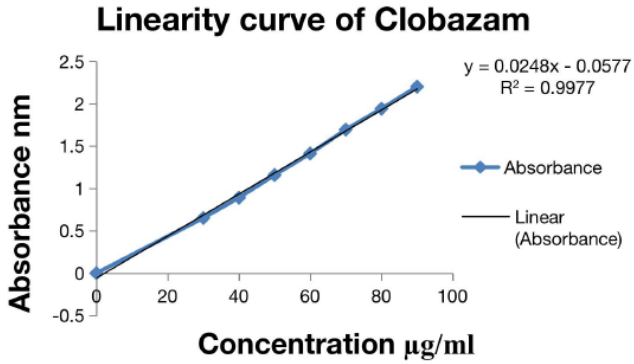
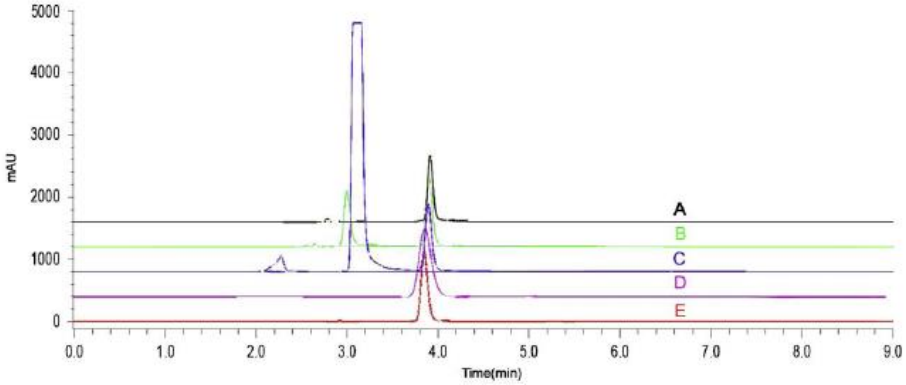


Figure: 3 Representative chromatogram of clobazam (50µg/mL), on Acidic (A), alkaline (B), Oxidative (c), Photolytic (D), and Thermal (E) degradation



DISCUSSION

The spectrophotometric method developed for the quantification of Clobazam in bulk and finished pharmaceutical preparations has proven to be highly accurate, sensitive, rapid, precise, and economical. Utilizing UV spectrophotometric absorption with a maximum absorbance at 230 nm, this method effectively addresses the need for a reliable analytical technique in the UV region of the spectrum. The sensitivity of the method is highlighted by the low Limit of Detection (LOD) and Limit of Quantification (LOQ), which were determined to be 0.2158 µg/ml and 0.6915 µg/ml, respectively. These values indicate the method's capability to detect and quantify very low concentrations of Clobazam, making it suitable for precise analytical purposes. A strong linear relationship was observed between 30-90 µg/ml when the calibration curve of Clobazam was plotted at 230 nm, with the regression equation Y = 0.0248x and a correlation coefficient of 0.997. This demonstrates excellent linearity and indicates that the method can accurately measure Clobazam across this concentration range. The method's accuracy was further validated by calculating the mean percentage recovery, which ranged from 99.50% to 100%. Such high recovery rates confirm the method's capability to measure the actual content of Clobazam without significant interference from other components. Precision studies revealed a Relative Standard Deviation (RSD) of 0.42% for intra-day and 0.56% for inter-day measurements, indicating consistent

results across different times of the day and different days. Similarly, the RSD values for accuracy and robustness studies were less than 2.0%, underscoring the method's precision, accuracy, and robustness. When applied to Clobazam tablets, the method yielded assay results of 99.97%, with RSD values remaining below 2.0%. This high degree of accuracy and precision confirms the method's suitability for routine analysis of Clobazam in tablet form. Further validation parameters showed a capacity factor greater than 2, theoretical plates numbering 8576 (well above the minimum requirement of 2000), and a tailing factor of 1.27 (less than 2), all of which indicate excellent chromatographic performance for the Clobazam peak. Robustness was demonstrated by maintaining % RSD values under original and varied conditions below 2.0%, indicating that minor changes in method parameters do not significantly affect the results. In degradation studies, Clobazam exhibited minimal degradation under thermal, oxidative, and UV conditions, with less than 6.0% decomposition. Acidic conditions led to 7.80% degradation, while alkaline conditions resulted in the appearance of a major degradant at 2.987 minutes without interfering with the main drug peak at 3.891 minutes, with a drug decomposition percentage of 2.85%. These findings highlight the method's capability to distinguish between the drug and its degradation products, ensuring accurate measurement even in stressed conditions.

CONCLUSION

The developed RP-HPLC method for the determination and validation of Clobazam in bulk and pharmaceutical dosage forms proves to be specific, linear, precise, accurate, and system suitable. The method development and optimization focused on enhancing final performance, making validation straightforward and effective for analyzing preclinical samples, formulations, and commercial samples. The validation parameters—accuracy, precision, specificity, and linearity—met ICH and USP requirements and complied with BEER'S law. Therefore, this method is reliable and can be routinely employed for the analysis of Clobazam in both bulk and tablet forms.

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