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Research Article

Application of High-Performance Thin Layer Chromatography in the Quantitative Estimation of Serum Clobazam Levels

Kulkarni-Munshi R*, Vishwakarma J

Dept. of Clinical Pharmacology, TN Medical College & BYL Nair Hospital, Dr. AL Nair Road, Mumbai Central, Mumbai 400 008, Maharashtra, India

ABSTRACT

This study was aimed to develop and validate a High-Performance Thin Layer Chromatography (HPTLC) method for the quantitative determination of serum clobazam levels. This simple and sensitive method will help in therapeutic drug monitoring that aids in the clinical management of patient therapy. Chromatographic separation was carried out on a silica gel 60F254 HPTLC plate using a mixture of Toluene:Methanol:Glacial acetic acid (15:1:0.16, v/v/v) as the mobile phase. Densitometric detection was carried out at 231 nanometers. The method was validated for specificity, precision, accuracy, robustness, linearity, limit of detection, and limit of quantification. Linear calibration curves in the range of 5-80 microgram per band gave a correlation coefficient of 0.99077. The intra-day (n=6) and inter-day (n=18) precision, expressed as the relative standard deviation were in the range of 0.36 to 4.44% and from 0.76 to 2.13%. Clobazam gave a well separated peak at Retardation factor (Rf) 0.30. Caffeine was used as an internal standard, which gave a well separated peak at Retardation factor (Rf) 0.20 without interfering with clobazam. The method was found to be specific with no matrix interference. Thus, the method developed for the estimation of serum clobazam level is simple, cost-effective and reliable for therapeutic drug monitoring.

Keywords: Clobazam; Caffeine; High-performance thin-layer chromatography; Therapeutic drug monitoring; Anti-epileptic drugs

INTRODUCTION

Benzodiazepines are used primarily as sedative-antianxiety drugs and have broad antiseizure properties. Various neuropsychiatric disorders including epilepsy, Huntington disease, addictions, sleep disorders and more are treated with benzodiazepines. Clobazam (CLB) is a 1,5-benzodiazepine which is an antiepileptic drug chemically known as 7-Chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4- [3H, 5H] dione. Clobazam is less acidic and less lipophilic with less sedation and it may be better tolerated than other 1,4-benzodiazepines [1]. When taken orally, within 1-4 hours, clobazam is well absorbed with peak concentration. It appears to give rise to a weaker tolerance than other benzodiazepines used as anticonvulsants as it acts to some extent like an agonist and has high therapeutic index with mild side-effects [2]. It is specifically useful when used concomitantly with other antiepileptic drugs, as it helps to prevent dependency [3]. Clobazam is sold in India under different brand names such as Clozam, Clobastar, Clodus, Lobazam and Frisium as an anxiolytic, anticonvulsant and antiepileptic drug [4]. Clobazam has a half-life of 18 h and is effective at doses between 0.5 and 1 mg/kg daily, with limited development of tolerance. The active metabolite of clobazam is norclobazam which has a half-life of 42 hours [1]. Clobazam is metabolized in the liver by demethylation and hydroxylation. It is excreted both as metabolized and unaffected, mainly in the urine [5].

Several studies have reported different methods for the determination of clobazam in urine as well as in serum using gas chromatography and high-performance liquid chromatography (HPLC) or high-performance liquid chromatography with a diode array detector (HPLC/DAD) [3, 5-7]. As clobazam has high therapeutic range, HPTLC method provides the advantage of detecting the precise concentration of clobazam available for biological activity in human serum. This paper presents the reliable and sensitive HPTLC method for the clobazam determination in human serum suitable for the bioavailability studies. This will help in the clinical management of patients receiving Clobazam in addition to other anti-epileptic drugs. Liquid- liquid phase extraction method was used to prepare the samples prior to the running of the analytic HPTLC system. An internal standard, caffeine was employed to control the extraction procedure.

Correspondence to: Dr. Renuka Kulkarni-Munshi, Professor (Addl) & Head In-charge, Dept. of Clinical Pharmacology, TN Medical College & BYL Nair Hospital, Dr. AL Nair Road, Mumbai Central, Mumbai 400 008, Maharashtra, India, Tel 91 22 2305 0347/2302 7204; E-mail: renuka.munshi@gmail.com

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EXPERIMENTAL

The reference standard of Clobazam was received from Sun Pharmaceuticals, Mumbai India, as a generous donation for the research. Caffeine was used as an internal stand¬ard and was purchased from Sigma-Aldrich (Mumbai, India). Chemicals like Toluene, Methanol and Glacial acetic acid (AR grade) were purchased from Merck (Darmstadt, Germany). Human blank serum used for development and vali¬dation of the procedure was obtained from healthy volunteers.

Equipment

The equipment used for method development and validation consisted of 100 µL Micro syringe (Linomat syringe, Hamilton-Bonaduz Schweiz all from CAMAG, Muttenz, Switzer-land), precoated silica gel 60F254 HPTLC plates (10 × 10 cm with 200 µm thickness HPTLC; Merck), Camag Linomat V automatic sample applicator (Camag), Camag twin trough chamber 10 × 10 cm (Camag), UV chamber (Camag), TLC scanner III (Camag), and winCATS version 1.4.9. software (Camag) were used in this study.

Chromatographic Conditions

Standard and sample solutions, 30 µL each, were applied on the TLC plate using Camag Linomat V automatic sample applicator in the form of band (bandwidth: 4 mm, distance between two bands: 14 mm) using micro syringe along with nitrogen aspirator was used for sample application; the samples were applied under constant flow rate to obtain a band length of 4 mm on the plate. A constant application rate of 150 nL/s was used. The plates were saturated for 20 minutes in a twin trough glass chamber (for 10 × 10 cm) with the mobile phase of toluene: methanol: glacial acetic acid (15:1:0.16, v/v/v). The plates were then placed in the mobile phase, and ascending development was performed up to a distance of 80 mm. Subsequent to the development, the plates were air dried and a densitometric scanning (slit dimensions: 4 × 0.45 mm) was performed at 231 nm using Camag TLC scanner III operated in reflectance-absorbance mode. WinCATS software was used to carry out the Spectral analysis and evaluation of the retention time of clobazam and internal standard and for further quantification of clobazam.

Preparation of mobile phase and stock solution

The method was developed using Toluene:Methanol:Glacial acetic acid (15:1:0.16, v/v/v) as the mobile phase and the precoated silica gel 60F254 TLC plates was used as stationary phase. Clobazam tablet was crushed into fine powder using mortar and pestle and used to prepare the stock solution. 10 milligrams of clobazam was weighed accurately and transferred to 10 mL volumetric flask with methanol as a diluent to get a concentration of 1 mg/mL. The stock solution of internal standard was prepared by dissolving 10 mg of 8-(3-Chlorostyryl) caffeine in 10 mL of Methanol. Aqueous trials of clobazam were prepared by making required dilutions of 1000 ng, 400 ng, 300 ng, 100 ng, 30 ng. 30 µL of dilutions were applied on TLC plates by using Camag Linomat IV Semiautomatic sample applicator and Hamilton syringe (100 µL). The stock solution of caffeine was prepared.

Preparation of Calibration Standards and Quality Control Samples

Working standard solutions for calibration and quality controls were prepared from secondary standard solution by dilution with methanol. The calibration standards were prepared by dissolving appropriate volume of stock solution of clobazam in drug-free human serum and further serial diluting it to provide a calibra¬tion range of 30 ng/band to 1000 ng/band in human serum. Quality control samples were prepared by spiking blank plasma with 30 ng/mL (LLOQ QC, lower limit of quantification quality control), 100 ng/mL (LQC, low quality control), 300 ng/mL (MQC-01, medium quality control), 400 ng/mL (MQC-02, medium quality control), 1000 ng/mL (HQC, high quality control) of clobazam. The inter¬nal standard was spiked in the serum before performing the extraction procedure.

Serum Sample Preparation

To 200 µL of serum sample (calibration standards, quality con¬trol samples, and patient samples), 10 µL of 1 mg/mL of inter¬nal standard was spiked and vortexed. 1000 µL of toluene was added as an extracting agent and liquid-liquid extraction was carried out. The mixture was vortexed for 30 seconds and then centri¬fuged at 12,000 rpm for 10 mins at 21°C. Under the stream of nitrogen, the supernatant was recovered and evaporated to dryness. The resi¬due was reconstituted in 80 µL of mobile phase.

Method Validation

The method was validated according to the International Con¬ference on Harmonization (ICH) guidelines using the following parameters: specificity, precision, accuracy, robustness, linearity, limit of detection, and limit of quantification [8-12].

Specificity: The measure of interference from other ingredients and degradation products is known as specificity [8]. To ensure that there is no interference at the retarda¬tion factor (Rf) of clobazam with that of the internal standard, the diluents used or the degradation products that may form during the extraction process, the specificity of the method was determined. Specificity of the method was probed by com¬paring chromatograms of both blank serum samples and spiked serum samples of the concentration range from 30 to 1000 ng, the diluents used, the internal standard, and the reference standard. To ensure the specificity of the spectra of the reference standard and the extracted clobazam samples, spectral analysis was performed.

Precision and Accuracy: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the given set of conditions is defined as precision of analytical procedure. It is expressed in terms of coefficient of variation or standard deviation. Precision is expressed at three levels intermediate precision, repeatability, and reproducibility. The closeness between the accepted value or a conventional true value or a reference value and the value obtained experimentally is defined as the accuracy of an analytical procedure which is also termed as trueness [13].

Precision and accuracy of the method were assessed by executing replicate analysis of the 5 quality control samples (30, 100, 300, 400 and 1000 ng/band, each concentration was prepared in three replicates, and each replicate was spotted thrice) against the calibration curve by carrying out the analytical run within the same day twice with 3 hour interval (intraday) and 6 days (interday) [8,9]. Calculation of precision was done by figuring out the percentage relative standard deviation for repeated measurements, and accuracy was expressed as % of recovery. Accuracy was determined results as the percentage of analyte recovered, determined by the calibration curve equation from the test [8, 10].

Robustness: The robustness of an analytical procedure is defined as a measure of its capacity to remain unaltered by small, but deliberate, variations in method parameters. It also provides a manifestation of its reliability during normal usage [8]. Robustness was performed by changing the ratio of the mobile phase ratio used as toluene: methanol: glacial acetic acid (15:1:0.16, v/v/v). Robustness of the method was carried out in triplicates and its mean %RSD (% relative standard deviation) was calculated.

Calibration curve: Each calibration curve contained a single set of calibration standards and six replicates of quality control (QC) at five concentration levels. The calibration curve was constructed by using the ratio of peak area of clobazam and internal standard

versus the con-centration added. The concentration range of 30 ng/band to 1000 ng/band was used. A linear relationship between peak area and concentration was evaluated. The coefficient of correlation was determined to assess the linearity of the calibration curves [8,9].

Detection and Quantification Limits: The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated with the help of calibration curve of three samples with low concentration. The limit of detection (LOD) was calculated using the formula 3.0 σ/S phenomena of the calibration curve, and the limit of quantification (LOQ) was calculated using the formula 10 σ/S phenomena of the calibration curve for the limits of detection and quantification, respectively, where σ is the standard deviation of the y-intercepts and S is the slope of the calibration curve [8, 10, 11].

RESULTS AND DISCUSSION

During method development, several trials were performed using different solvents with varying polarity and in different proportions to obtain good resolution and sharp peaks with acceptable Rf values (0.2-0.8). Among different mobile phase, combinations were tested, mobile phase consisting toluene: methanol: glacial acetic acid (15:1:0.16, v/v/v) gave better resolution and sharp peaks with saturation time of 20 minutes.

Specificity

The specificity of the method is illustrated in Figure 1 where there is complete separation of peaks of interest at Rf obtained with blank plasma and plasma spiked with clobazam. In addition, there was no interference at the Rf of clobazam in the tablet form. Clobazam and the internal standard (Caffeine) were efficiently separated and detected at the Rf of 0.30 ± 05 and 0.20 (± 0.03) indicating the specificity of the method.

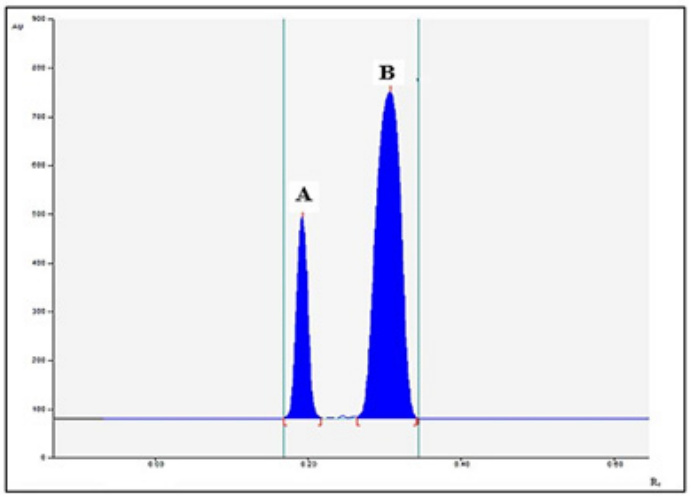

Figure 1: Spectral analysis and evaluation of the retention time of clobazam and internal standard was done by WinCATS software. Clobazam gave a well separated peak at Retardation factor (Rf) 0.30. Caffeine was used as an internal standard, which gave a well separated peak at Retardation factor (Rf) 0.20 without interfering with clobazam at 231 nm.

Precision and Accuracy

Precision and accuracy of the method were assessed by executing replicate analysis of the 5 quality control samples prepared in three replicates, and each replicate was spotted thrice against the calibration curve by carrying out the analytical run within the same day twice with 3-hour interval (intraday) and 6 days (inter-day). The relative standard deviation (RSD) for precision ranged from 0.82 to 1.98 and from 1.5 to 2.51 for intra- and inter-day analyses, respectively (Table 1). Accuracy was calculated and was found to be between 94.8-104.9% and 97.8-100.83% for intra-day and inter-day analysis, respectively. The same has been expressed as analyte recovery percentage (Table 2).

Concentration [µg/band]	RSD intraday (n = 6)	RSD inter-day (n = 18)
30	0.82	1.5
100	1.53	1.73
300	1.06	1.99
400	1.83	1.57
1000	1.98	2.51
µg- microgram, RSD-Relative standard deviation		

Table 1: Precision of Clobazam in serum

Concentration [µg/band]	Calculated concentration (mean ± SD)	Recovery %
Intra-day (n = 6)		
30	30 ± 0.29	98.15
100	100 ± 0.45	94.8
300	300 ± 1.13	104.9
400	400 ± 1.07	100.9
1000	1000 ± 1.53	99.2
Inter-day (n = 18)		
30	30 ± 0.28	100.83
100	100 ± 0.47	97.83
300	300 ± 1.25	99.28
400	400 ± 1.51	97.8
1000	1000 ± 2.42	98.13
µg-microgram, SD-Standard deviation		

Table 2: Accuracy of Clobazam in serum

Calibration curve

The calibration curve was found to be linear; the coefficient of correlation was found to be 0.9963 with the standard deviation of 12.37% (Figure 2).

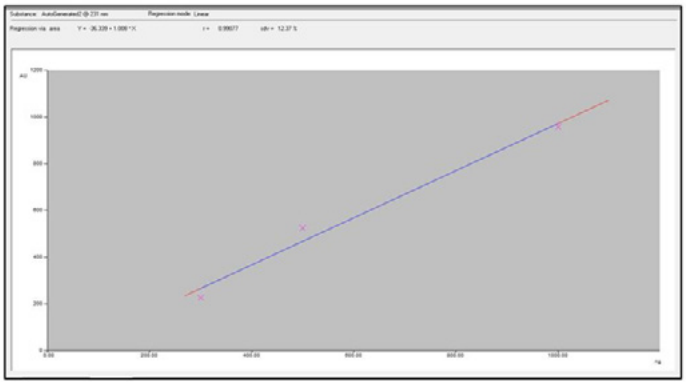

Figure 2: Calibration curve of Clobazam in serum. Linear Calibration curve was observed with coefficient of correlation–0.9963 and standard deviation of 12.37% of Clobazam in serum.

Detection and Quantification Limits

The lowest limit of detection (LOD) and the lowest limit of quantification (LOQ) were found to be 5 µg/band and 80 µg/band respectively, which are well within the therapeutic needs.

APPLICATION

This is a therapeutic drug monitoring (TDM) application for analysis of clobazam with HPTLC method. The validated method was applied to perform the drug analysis of 42 patients on oral dose of clobazam tablets. The blood was collected and centrifuged at 12,000 rpm for 10 minutes to separate serum. The serum was frozen properly prior to analysis. After carrying out the analysis of these 42 patients samples, it was found that the quantification values of 29 patients samples were well within the therapeutic range, while those of 9 patients were below the therapeutic range and above the therapeutic range in case of 5 patients. The drug levels were correlated with the medical history and current clinical condition of the patients, and it was found that 4 of the 9 patients in whom the drug level was below the therapeutic range were consuming doses lower than prescribed.

CONCLUSION

The demand for HPTLC is increasing at a high rate in pharmacopeias and good manufacturing practices (GMPs) for botanical dietary supplements, and it is predicted that the use of HPTLC methods on synthetic drugs will also increase in worldwide pharmacopeias. HPTLC procedures are highly variegated, based on the actual type of test; it can be used in a broad range of applications from simple qualitative identification to quantitative assay or purity test [8].

A guide towards maintaining optimal blood concentrations of antiepileptic drugs that optimize seizure control, while avoiding or at least minimizing toxic effects is known as therapeutic drug monitoring (TDM) of anti-epileptic drugs. The therapeutic drug monitoring (TDM) application for analysis of clobazam was done with HPTLC method. With respect to the importance of TDM of clobazam, a broad range of analytical approaches has been documented for the measurement of clobazam in blood including gas chromatography (GC), GC/mass spectrometry (MS), high-performance liquid chromatography (HPLC) and high-performance liquid chromatography with diode array detectors (HPLC/DAD) which are highly expensive. The proposed method is specific, precise, accurate, reproducible and rapid and could be used for routine analysis of clobazam in serum for bioanalytical studies. This method offers remarkable advantage over most of the analytical methods reported for quantification of clobazam in biological fluids, in terms of simplicity of extraction procedure and overall analysis time. The method also has a good linearity, specificity and is also suitable for high throughput clinical sample analysis. There are very few documented reports on therapeutic drug monitoring that using HPTLC can be a comparatively less expensive technique. HPTLC provides the potentiality to run 10-20 samples at the same time in a single run. It also requires less volume of mobile phase when compared to other HPLC methods on clobazam. Accordingly, we developed this method for the determination of serum clobazam level using HPTLC which is reliable, and reproducible. It is simple, as it involves simple liquid–liquid extraction using toluene which gives 950 µL of sample on extraction. Therefore, this validated method is acceptable for the quantitative determination of clobazam in human serum as well as pharmacokinetic and bioavailability studies of clobazam.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Brunton LL, Dandan RH, Knollmann BC. Goodman and Gilman's The Pharmacological Basis of Therapeutics, 13th Ed., McGraw-Hill, New York, 2018.
2. Akerman KK. Analysis of clobazam and its active metabolite norclobazam in plasma and serum using HPLC/DAD.
3. Scand J Clin Lab Invest. 1996;56(7):609-614.
4. Bolner A, Tagliaro F, Lomeo A. Optimised determination of clobazam in human plasma with extraction and high-performance liquid chromatography analysis. J Chromatogr B. 2001;750(1):177-180.
5. Medicine Brands for Generic 'Clobazam'. Medicine India. 920.
6. Christian S, Patel R, Shivprakash. A rapid and sensitive LC-MS/MS assay for the determination of clobazam in human plasma using electro spray ionization technology. Int J Pharm Sci Res. 2018; 9(6):2369-2377.
7. Ashton H (1994). Guidelines for the Rational Use of Benzodiazepines: When and What to Use. Drugs. 1994;48(1):25-40.
8. Gazdzik WR, Podlesany J, Filipek M. HPLC method for simultaneous determination of clobazam and N desmethylclobazam in human serum, rat serum and rat brain homogenates. Biomed Chromatogr. 1989;3(2):79-81.
9. Rashmin P, Mrunali P, Nitin D, Nidhi D, Bharat P. HPTLC method development and validation: Strategy to minimize methodological failures. J Food Drug Anal. 2012;20(4):794-804.
10. Shabir GA, John Lough W, Arain SA, Bradshaw TK. Evaluation and application of best practice in analytical method validation. J Liq Chromatogr R T. 2007;30(3):311-333.
11. Ferenczi-Fodor K, Vegh Z, Nagy-Turak A, Renger B, Zeller M. Validation and quality assurance of planar chromatographic procedures in pharmaceutical analysis. J AOAC Int. 2001;84(4):1265-1276.
12. Renger B, Vegh Z, Ferenczi-Fodor K. Validation of thin layer and high performance thin layer chromatographic methods. J Chromatogr A. 2011;1218(19):2712-2721.
13. Souri E, Farahani AD, Ahmadkhaniha R, Amini M. A stability indicating HPLC method for the determination of clobazam and its basic degradation product characterization. Daru. 2014;22(1):49.
14. Bala R, Khanna S, Pawar P, Arora S. HPLC method development and validation for the analysis of clobazam in rabbit plasma. World J Pharm Sci. 2016;5(6):1755-1762.