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A RAPID AND SENSITIVE LC-MS/MS ASSAY FOR THE DETERMINATION OF CLOBAZAM IN HUMAN PLASMA USING ELECTRO SPRAY IONIZATION TECHNOLOGY

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ABSTRACT: A simple, highly sensitive, precise and accurate liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed for the rapid quantification of clobazam in human plasma samples. The chromatographic separation was achieved with a reverse phase column repositol gold XBD C18 (100 × 3mm, 3µm) and the mobile phase consisted of 10 mM ammonium acetate buffer and 0.1% acetic acid in methanol (15:85, v/v) as eluent by running a linear gradient method. The effluence was ionized by positive electrospray ionization and measured by mass spectrometry. The method was validated with linear calibration curve over a wide dynamic concentration range of 0.501-499.995 ng/mL and the lower limit of quantification was 0.501 ng/mL with good accuracy and precision. The mean extraction recovery of the method was higher than 96.4% and 103.3% for clobazam and IS, respectively. The method was successfully demonstrated for evaluation of bioequivalence study of 10 mg clobazam tablet formulation in 28 healthy Indian male subjects under fasting condition.

INTRODUCTION: Clobazam, 7-chloro-1-methyl-5-phenyl-1, 5-benzodiazepine-2, 4 (3H-dione) (Figure 1), is a benzodiazepine derivative which is used for the treatment of various seizure types and epilepsy¹. Clobazam is used for sedation and as an antiepileptic drug, presenting some advantages over 1,4-benzodiazepines and anticonvulsant properties². Clobazam is metabolized in the liver by demethylation and hydroxylation.

It is excreted both unchanged and as metabolized, mainly in the urine. Several studies reported the effectiveness of clobazam in the treatment of partial and generalized seizures. Clobazam is only prescribed if first line antiepileptic such as phenytoin is unable to control seizures and is taken by mouth once daily to prevent the occurrence of epilepsy.

This once daily administration is possible due to long 18 hrs half life of clobazam and more likely to lead to patient compliance in epileptic attack as compared to phenytoin to be taken up to four times per day^{3, 4}. The use of LC coupled with electrospray tandem mass spectrometry has become a very popular technique in bioavailability studies due to the fast, sensitive and reliable results generated by its use.

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UPLC has been evaluated as a faster and more efficient analytical tool compared with current HPLC⁵.

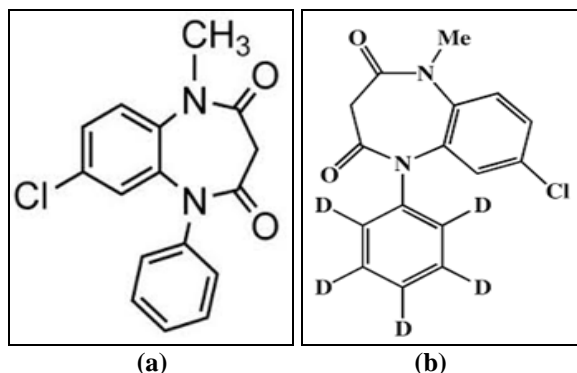


FIG. 1: CHEMICAL STRUCTURE OF CLOBAZAM (a) AND CLOBAZAM D5 (b)

Different analytical methods for determination of clobazam drug have been reported, including high performance liquid chromatography (HPLC) with UV detector^{6,7} using 1.0 mL of plasma aliquot, having lower detection 5.0 ng/mL and 100 μ l of injection volume. One method with direct injection of HPLC method by Pistos and James⁸ with lower detection 30 μ g/mL is reported. Method on HPLC with diode array detector by Akerman⁹ in human plasma and serum using solid phase extraction method is reported. In human urine rapid resolution by liquid chromatography - tandem mass spectrometry by Ren *et al.*,¹⁰ is reported where for clobazam is retained at 11.09 minute. Quantitation of benzodiazepines by Marin *et al.*,¹¹ and George *et al.*,¹² and screening method for benzodiazepines by Marion *et al.*,¹³ with LC-MS/MS are reported. As far as we are aware not a single method available to quantify individual clobazam (benzodiazepine drug) in human plasma using instrument HPLC connected with tandem mass spectrometer. Moreover these reported methods have higher quantification limit, longer chromatographic run time and larger aliquot volume which is normally not preferable when large number of samples are to be analyzed. Hence, it is necessary to develop and validate a simple, rapid and sensitive method which can be successfully applied to a bioequivalence study.

In this paper we describe a rapid, selective, and sensitive HPLC-MS/MS method for determination clobazam in human plasma. The method is extensively validated as per US Food and Drug Administration (FDA) guidelines¹⁴.

The validated method requires only 0.4 mL human plasma for liquid-liquid extraction (LLE) and demonstrated excellent performance in term of ruggedness and efficiency (4.0 min per sample). Interference due to matrix was ascertained by post column infusion technique. The method has been successfully used in a pharmacokinetic study approved by the appropriate ethics committee and performed on 28 healthy Indian males after obtaining their written informed consent. More than fifteen hundred samples were assayed by use of the proposed method. The method is not only selective and sensitive but faster and simpler.

MATERIALS AND METHODS:

Chemicals and Reagents: Clobazam and clobazam D5 working standards were procured from Synchron Research Services Pvt. Ltd. (Ahmedabad, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC methanol and acetonitrile were purchased from Merck (Mumbai, India). Ammonium acetate was purchased from Merck (Germany). Other reagents were used by analytical grade. Drug-free (blank) human plasma was obtained from Synchron Research Services Pvt.Ltd (Ahmedabad, India) and was stored at -70°C prior to use.

Preparation of Calibration Curve (CC) Standards and Quality Control (QC) Samples:

A stock solution of clobazam and internal standard (clobazam D5) were prepared individually in methanol at a concentration of 1 mg/mL. Working standard solutions for calibration and quality controls were prepared from secondary standard solution by dilution with water:methanol (1:1, v/v). The stock solutions were stored at -70°C; intermediate stock and working stock solutions were stored at 2-8 °C until use. Calibration standards were prepared by spiking blank plasma with clobazam to get the concentration of 0.501, 1.002, 25.052, 74.782, 249.271, 374.846, 449.995 and 499.995ng/mL. Quality control samples were prepared by spiking blank plasma with 400.235 ng/mL (HQC, high quality control), 248.146 ng/mL (MQC-01, medium quality control), 32.259 ng/mL (MQC-02, medium quality control), 1.290 ng/mL (LQC, low quality control) and 0.503 ng/mL (LLOQ QC, lower limit of quantification quality

control) of clobazam. The stability studies for quality control samples (HQC and LQC) spiked were stored at -70 °C.

Sample Preparation: A fifty microliters of the internal standard solution (376.109 ng/mL) was added to 0.4 mL of plasma, than vortex mixed for 30 s. Afterwards, 50 µL of 0.5% formic acid and 3 mL of methyl tert-butyl ether were added and vortex mixed for 5 min using multi-pulse vortexer (Glas-col, Terre haute, USA). Following centrifugation at 4000 rpm for 5 min, take out organic layer in to evaporation test tube and evaporated to dryness in a water bath at 50°C under a nitrogen stream. The residue was redissolved in 250 µL of mobile phase. An aliquot of 10 µL was injected into the LC–MS/MS system.

LC-MS/MS Method: LC/ESI-MS/MS was performed using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer connected to a Shimadzu UPLC™ system (Nexera-X2 SIL-30AC). An analytical Reprosil Gold XBD C18 (100×3 mm, 3µm) column was used with a mobile phase 10mM ammonium acetate buffer (15%) and 0.1 % acetic acid in methanol (85%). The flow rate gradient conditions are described in **Table 1**. The column was maintained at 40°C in column oven and auto-sampler temperature was maintained at 10°C. The total run time for an LC-MS/MS analysis was 4.0 min and injection volume was 10µL. Mass spectrometric detection was performed using a shimadzu LCMS-8040 triple quadrupole mass spectrometer with electro spray ionization (ESI) in the positive ion mode. The MS conditions were as follows: interface voltage: 4.5 kV, Q1 pre-rod bias voltage: 14 V, Q3 pre-rod bias voltage: 25 V, collision energy: 19 eV (KPA), nebulizer gas flow rate: 3 L/min, drying gas flow rate:15 L/min, desolvation line temperature: 250°C, heat block temperature: 400°C and collision gas: 230 kPa. Lab Solutions software (version 5.72B, Shimadzu) was used for the system control and data processing.

TABLE 1: HPLC GRADIENT FLOW PROGRAM

Time (min)	Pump Flow	%A	%B
0.01	0.20	15	85
1.50	0.20	15	85
1.51	0.60	15	85
3.70	0.60	15	85
3.71	0.20	15	85
4.00	0.20	15	85

Validation: The complete method validation of clobazam in human plasma was determined as per the US Food and Drug Administration (FDA) guidelines¹⁴. Each calibration curve contained a single set of calibration standards and six replicates of QC at five concentration level. Selectivity was assessed, by comparing the chromatograms of eight different batches including haemolysed and lipemic of blank plasma obtained from eight different sources (or donors) with those of corresponding standard plasma samples spiked with clobazam and clobazam D5. Sensitivity was determined by analyzing eight replicates of blank human plasma & plasma spiked with lowest level of the calibration curve. For the determining of intra-day accuracy & precision a replicates (n=6) analysis of plasma samples was performed on the same day. The inter-day accuracy & precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD %) and the accuracy as the relative error (RE %). Recovery was determined at low (LQC), middle (MQC-01) and high (HQC) quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 376.109 ng/mL. The matrix of plasma constituents over the ionization of analyte and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations^{15,16}.

Matrix effect was determined at two levels (HQC and LQC) with eight different source including haemolysed and lipemic of plasma for clobazam and clobazam D5. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Five replicates each at a concentration of double the uppermost calibration standard 999.990 ng/mL (2×ULOQ) were diluted 5-fold (199.998 ng/mL) and 10-fold (99.999 ng/mL) with blank plasma. The diluted samples were processed and analyzed.

Process sample stability was evaluated with freshly spiked calibration curve and quality control samples and those freshly spiked quality control samples compared with re-injecting the same sample which (stability samples) were stored at 10°C for 56h and room temperature (25 ± 5 °C) for 7h. Bench top stability was evaluated for 6h at

room temperature and compared with freshly spiked plasma samples. The freeze-thaw stability was determined by comparing the stability samples that had been frozen and thawed five times, with freshly spiked quality control samples. Long term stability was evaluated by analyzing at low (LQC) and high (HQC) quality control sampled that was stored at -70°C for 86 days together with freshly spiked calibration & quality control standards. All stability evaluations were based on back calculated concentrations.

Pharmacokinetic Study: The method was applied to determine the plasma concentrations of clobazam from a bioequivalence in which 28 healthy male volunteers received one tablet (containing 10 mg clobazam). The ethics committee approved the protocol and the volunteers provided with informed written consent to participate in the study according to the principles of the Declaration of Helsinki. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA¹⁷. Blood samples were obtained following oral administration of 10mg of clobazam tablet into K_2EDTA vacutainer solution as an anticoagulant at pre-dose, 0.50h, 0.75h, 1.00h, 1.33h, 1.67h, 2.00h, 2.33h, 2.67h, 3.00h, 3.33h, 3.67h, 4.00h, 4.50h, 5.00h, 6.00h, 8.00h, 10.00h, 12.00h, 16.00h, 20.00h, 24.00h, 48.00h, 72.00h, 96.00h, 120.00h and 144.00h. Plasma was separated by centrifuging the blood using thermo centrifuge 5810R (Eppendorf, Germany) at 4000 rpm for 10 min at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and stored frozen at $-70 \pm 10^{\circ}\text{C}$ until analysis. An aliquot of $400\mu\text{L}$ of thawed plasma samples were spiked with IS and processed. Plasma

concentration–time data of clobazam was analyzed by non-compartmental method using Kinetica Version 4.4 (Thermo USA). Regarding AUC_{0-t} , $\text{AUC}_{0-\infty}$ and C_{max} , bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data).

RESULT AND DISCUSSION:

Method Development: During method development different options were evaluated to optimize detection parameters, chromatography and sample extraction.

Mass Spectra: LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. Mass spectrometric detection was performed using a LCMS-8040 triple quadrupole mass spectrometer from Shimadzu, equipped with an electrospray ion source in positive ionization mode. Nitrogen was used as both the nebulizing and the desolvation gas, and argon was used as the collision gas. The collision energies were optimized at 19 KPa for Clobazam and 21 KPa for IS. The full scan spectrum was dominated by deprotonated molecules $[\text{M}+\text{H}]^+$ m/z 301.15 and 306.00 for clobazam and IS, respectively and major fragment ions observed in each product spectrum were at m/z 259.10, 264.05 for clobazam and IS, respectively. The mass spectra of precursor ion and product ion can be observed with most abundant intense peak in **Fig. 2** and **3** for clobazam and clobazam D5, respectively.

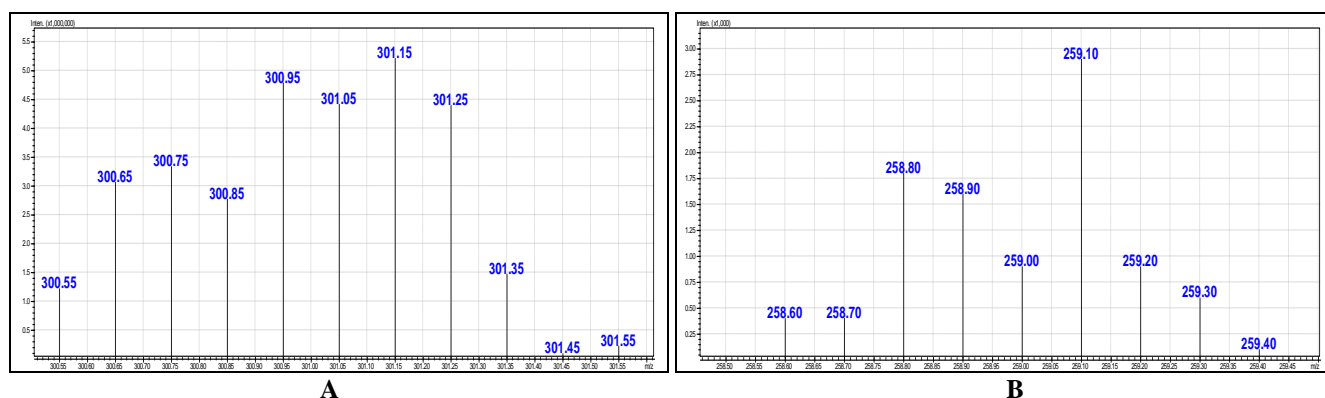


FIG. 2: ELECTROSPRAY POSITIVE ION MASS SPECTRA OF PRECURSOR ION (a) AND PRODUCT ION (b) OF CLOBAZAM

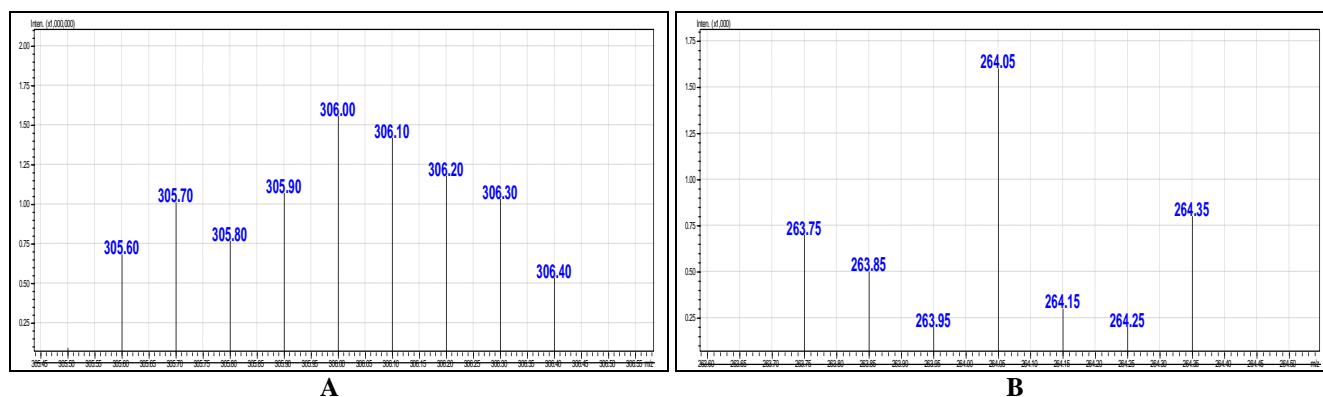


FIG. 3: ELECTROSPRAY POSITIVE ION MASS SPECTRA OF PRECURSOR ION (a) AND PRODUCT ION (b) OF CLOBAZAM D5

Chromatography: The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. Initially, a mobile phase containing formic acid solution and acetonitrile in varying combinations was tried in which poor peak shape was observed. The mobile phase containing 0.1% acetic acid in water: acetonitrile (20:80v/v) and 0.1% acetic acid in water: methanol (20:80 v/v) was tried but still desirable peak shape was not observed. Later the mobile phase containing water:0.1% acetic acid in acetonitrile (20:80 v/v) and water:0.1% acetic acid in methanol (20:80 v/v) exhibited better separation, but response was very low, which was insufficient to quantify LLOQ (0.501 ng/mL). To improve peak shape and enhance response we had tried mobile phase 10 mM ammonium acetate solution in combination with 0.1% acetic acid in methanol and acetonitrile in varying combinations.

We had also tried 2 mM and 5 mM ammonium acetate solution. Finally we received the best signal for clobazam and clobazam D5 using a mobile phase containing 10 mM ammonium acetate solution in combination with 0.1% acetic acid in methanol (15:85 v/v) as there was almost a threefold increase in its area count observed. Moreover, a marked improvement in the peak shape of clobazam and clobazam D5 was also observed using this mobile phase.

Columns such as, Hypersil gold C18 (100 mm x 2.1 mm, 4 μ m), Gemini-NX (50 mm x 4.6 mm, 5 μ m), orosil C18 (150 mm x 4.6 mm, 3 μ m), Chromatopak (50 mm x 4.6 mm, 5 μ m) and Reprosil Gold XBD C18 (100 mm x 3 mm, 3 μ m)

were tried during the method development. In Hypersil C18 and Gemini-NX C18 columns poor chromatography was observed. The best results were achieved using the Reprosil Gold XBD C18 column. It gave satisfactory peak shapes for both clobazam and IS and a gradient flow rate reduced the run time to 4 min. The column oven temperature was kept at a constant temperature of about 40°C.

Under mentioned conditions the chromatographic analysis of standard solution containing the present analyte showed a good separation of clobazam with retention time of 2.30 min and clobazam D5 with retention time of 2.30 min, making the run time only 4.0 min and this met the requirement for a high sample throughput. A simple liquid-liquid extraction (LLE) was used in assay.

Extraction: Prior to LC injection, the co-extracted proteins should be removed from the prepared solution. Several organic solvents were employed to extract analyte from the plasma sample. Out of the tested solvents (methyl tertiary butyl ether, ethyl acetate, dichloromethane, chloroform, hexane, acetonitrile) methyl tert-butyl ether extract more effectively than other solvents. So the method was optimized to achieve maximum extraction efficiency. The high recovery and selectivity was observed in the liquid-liquid extraction method which was used in the present work.

These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of clobazam in matrix.

Specificity and Selectivity: Eight different lots of plasma along with one lipemic plasma and one haemolyzed, plasma were analyzed to ensure that no endogenous interferences were present at the retention time of clobazam and IS. Eight LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analyzed.

In all plasma blanks, the response at the retention time of clobazam was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. As shown in **Fig. 4** no interference peaks from endogenous compound is observed at the retention times of analyte and IS.

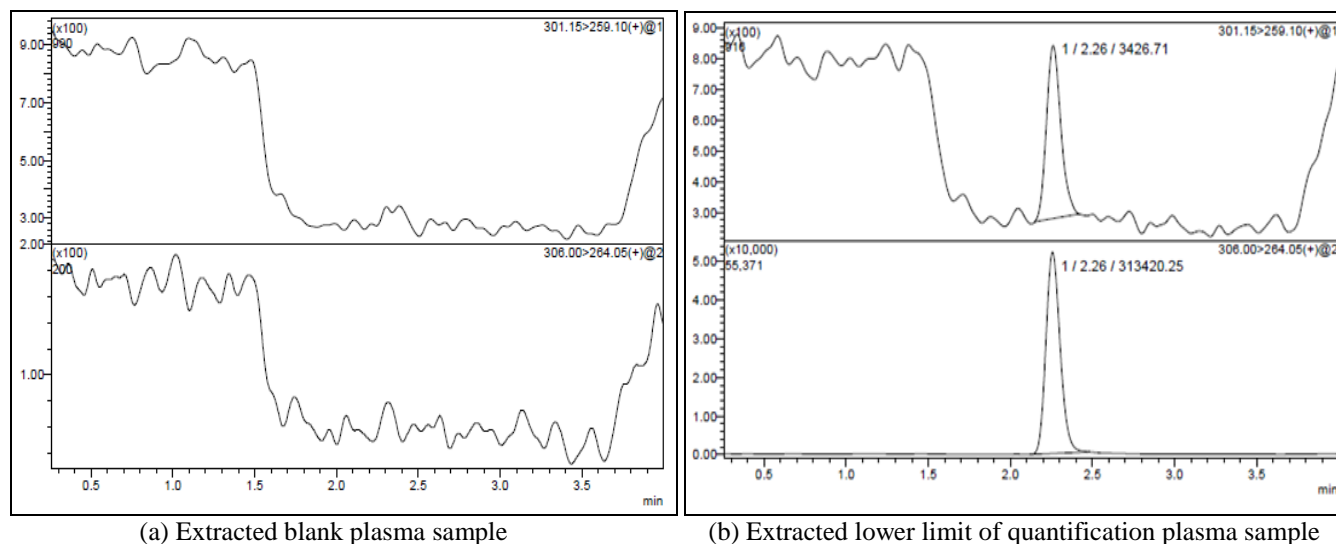


FIG. 4: REPRESENTATIVE CHROMATOGRAMS OF (a) EXTRACTED BLANK PLASMA SAMPLE; (b) EXTRACTED LOWER LIMIT OF QUANTIFICATION PLASMA SAMPLE

Linearity: The plasma calibration curve was constructed using calibration standards 0.501-499.995 ng/mL for clobazam. Linearity was assessed by a weighted ($1/c^2$) least squares regression analysis. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/c^2$ weighing factor, giving a

mean linear regression equation for the calibration curve of: $y = mx + c$ for clobazam where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.99 or better; **Table 2** summarizes the calibration curve results.

TABLE 2: PRECISION AND ACCURACY DATA OF BACK-CALCULATED CONCENTRATION OF CALIBRATION SAMPLES FOR CLOBAZAM IN HUMAN PLASMA

Analyte	Concentration added (ng/mL)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)
Clobazam	0.501	0.505	1.19	0.80
	1.002	0.982	2.34	-2.00
	25.052	25.555	1.59	2.01
	74.782	75.120	0.95	0.45
	249.273	248.241	0.85	-0.41
	374.846	370.559	0.67	-1.14
	449.995	461.011	0.50	2.45
	499.995	488.422	1.52	-2.31

To determine weighting factor for clobazam calibration standards accuracy results were calculated for three P&A batches with linear regression ($y=mx+c$) using weighting factors as $1/C$, $1/C^2$ and no weighting. The percent relative error (%RE) were calculated for each standards level as percent difference between the observed value of all %RE are summed for each weighting

factor in three calibration standards. The weighting factor that gives the smallest values of the sum of %RE is taken as concentration response relationship for clobazam. In our case it is $1/C^2$.

The $\Sigma\%RE$ for weighting factor none, $1/C$ and $1/C^2$ for clobazam is 758.1 %, 42.6 % and 36.3 % respectively.

Sensitivity: The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and it was found to be 0.501 ng/mL for clobazam.

The intra-day precision at the LLOQ was 2.0%, and the intra-day accuracy was 7.0% for clobazam **Table 3.**

TABLE 3: PRECISION AND ACCURACY OF THE METHOD FOR DETERMINING CLOBAZAM CONCENTRATION IN PLASMA SAMPLES

Analyte	Intra-day precision(n=6)				Inter-day precision (n=18)		
	Concentration added (ng/mL)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)
Clobazam	0.503	0.505	2.0	0.4	0.502	7.0	-0.2
	1.290	1.299	5.2	0.7	1.279	3.8	-0.9
	32.259	31.059	0.8	-3.7	30.987	0.8	-3.9
	248.146	246.439	0.5	-0.7	244.971	0.7	-1.3
	400.235	405.437	0.4	1.3	400.791	1.1	0.1

Precision and Accuracy: The results for intra-day and inter-day precision and accuracy for clobazam in plasma quality control samples are summarized in **Table 3.** The intra-day precision and accuracy was $\leq 5.2\%$ and $\leq 7.0\%$. The inter-day precision and accuracy was $\leq 3.7\%$ and $\leq 3.9\%$.

Recovery: Six aqueous replicates (samples spiked in mobile phase) at low (LQC), middle (MQC-01) and high (HQC) quality control concentration levels for clobazam were prepared for recovery determination and the areas obtained were compared versus the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery was 96.4% and 103.3% for clobazam and IS, respectively. The observed variability in % CV between these levels was 2.1% for clobazam and 2.0% for IS. The recovery obtained for analyte was higher than that of other reported methods.

Matrix Effect: The assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS for supporting pharmacokinetics studies. The matrix effect was evaluated by analyzing QC samples. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained were +0.98 (CV 2.8%, n= 8) at LQC and +1.01 (CV 2.5%, n= 8) at HQC level for clobazam, whereas on IS it was found to be +0.96 (CV 2.7%, n= 8) at tested concentration of 376.109 ng/mL. Matrix effect was not observed at analytes and IS retention times.

Dilution integrity: The upper concentration limits could be extended to ($2 \times \text{ULOQ}$) 999.990 ng/mL for clobazam by a 5-fold or 10-fold dilution with human plasma. The nominal concentration (%) for 1/5 and 1/10 dilution samples ranged from 199.998ng/mL and 99.999 ng/mL, respectively. The accuracy for 1/5 and 1/10 dilution samples 100.40 and 98.70, respectively. The coefficient of variation for 1/5 and 1/10 dilution samples of clobazam were 0.9% and 0.6%, respectively.

Stability and Incurred Sample Reanalysis: Exhaustive experiments were performed to assess the stability of clobazam in stock solution and in plasma samples under different conditions simulating the conditions occurring during analysis of study samples—room-temperature stability, extracted sample stability (process stability), freeze–thaw stability, and long-term stability of plasma samples. The results obtained were well within acceptable limits. IS stock solution was also found to be stable. Stock solutions of clobazam and IS were stable at room temperature for 7 h and at 2–8°C for 33 days. Clobazam in control human plasma were stable for 7 h at room temperature and extracted plasma samples were stable for 78 h in an autosampler at 10°C. Freeze and thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability of clobazam. Long-term stability of the analyte in plasma at -70 °C was found to be stable for at least 86 days at -70 °C. Percentage changes of concentration in these stability experiments are listed in **Table 4.**

TABLE 4: STABILITY SAMPLES RESULT FOR CLOBAZAM

Stability	n	Spiked Concentration (ng/mL)	Mean calculated comparison sample concentration (ng/mL)	Mean calculated stability sample concentration (ng/mL)	Mean percentage change
Process/Wet extract ^a	6	1.290	1.260	1.321	4.8
	6	400.235	405.437	422.426	4.2
Process/Wet extract ^b	6	1.290	1.115	1.156	3.7
	6	400.235	378.013	362.231	-4.2
Bench top ^c	6	1.290	1.349	1.281	-5.0
	6	400.235	403.863	398.409	-1.3
Freeze and thaw ^d	6	1.290	1.260	1.307	3.7
	6	400.235	405.437	412.614	1.8
Freeze and thaw ^e	6	1.290	1.260	1.302	3.3
	6	400.235	405.437	415.164	2.4
Long-term ^f	6	1.290	1.287	1.265	-1.8
	6	400.235	404.531	412.102	1.8

After 78 h in auto sampler at 10°C; ^b After 7 h in room temperature (25±5°C); ^c After 6 h at room temperature; ^d After five freeze and thaw cycles at -20°C; ^e After five freeze and thaw cycles at -70°C; ^f At -70°C for 86 days

An incurred sample re-analysis (ISR) was also conducted by computerized random selection of subject samples, 10 % of total no of subject samples, which us 144 samples analyzed. The selection criteria included samples which were near the C_{max} and the elimination phase which is having concentration at least 3 times of LLOQ in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than $\pm 20\%$ ¹⁸.

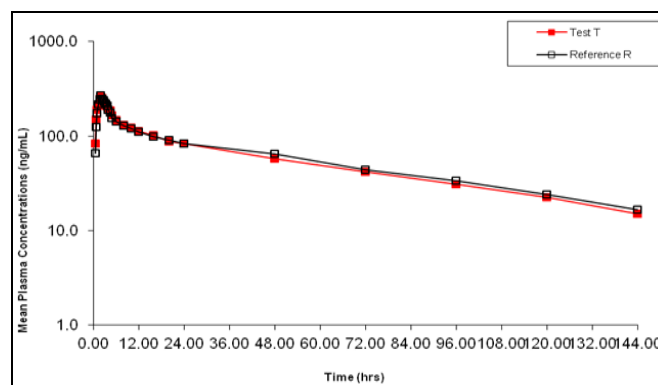
Pharmacokinetic Study: The proposed method was applied to determine clobazam levels in plasma for a bioequivalence study in 28 healthy Indian male volunteers who were orally administered 10 mg clobazam in tablet form. High-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples, and the described LLE method of sample preparation is suitable for this purpose.

TABLE 5: BIOEQUIVALENCE PARAMETERS OBTAINED AFTER ORAL ADMINISTRATION OF REFERENCE DRUG AND TEST DRUG AT THE CLOBAZAM DOSE OF 10 MG

Pharmacokinetic Parameters (Clobazam)	Test A	Reference
T_{max} (h)	2.06 ± 0.84	1.92 ± 0.73
C_{max} (ng/ml)	315.360 ± 92.58	309.865 ± 65.55
AUC ₀₋₁₄₄ (h·ng/ml)	7760.255 ± 1859.64	7985.797 ± 2023.66
AUC _{0-inf} (h·ng/ml)	9462.221 ± 2221.53	9512.761 ± 2951.34
$t_{1/2}$ (h)	57.224 ± 31.10	50.545 ± 17.31

Fig. 5 shows the mean plasma concentration-time curves for the two formulations. Pharmacokinetic

parameters derived from these curves are presented in **Table 5**. No significant differences were observed between the two formulations in terms of; C_{max} , AUC_{120h}, AUC_{inf}, T_{max} or $t_{1/2}$.

**FIG. 5: MEAN PLASMA CONCENTRATION-TIME PROFILE OF CLOBAZAM IN HUMAN PLASMA FOLLOWING ORAL DOSING OF 10MG CLOBAZAM TABLET TO 28 SUBJECTS**

CONCLUSION: The LC-MS/MS method described for clobazam was simple, rapid, reproducible and suitable for their determination in human plasma. The method offers significant 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. The method was selective in the presence of medications commonly used by human volunteers. Ion suppression/enhancement was studied by the post column infusion of analyte and post extraction spiking technique.

This validated method is suitable for the quantitative determination of clobazam in human plasma as well as pharmacokinetic and bioavailability studies of clobazam.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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