INTRODUCTION

Acute seizures are a common occurrence in the intensive care unit (ICU), either as a primary reason for admission or as a neurologic complication of a severe metabolic disorder or critical illness state. Rapid identification of acute seizures must be followed by immediate treatment with a fast-acting antiepileptic drug (AED). This is essential to reduce potential sequelae, particularly ischemic and excitotoxic neuronal cell loss, which begins within minutes of continuous seizure activity [1]. Fast-acting benzodiazepines, second-line AEDs such as phenytoin/ fosphenytoin, sodium valproate, lacosamide, [3] or the SV2A AED levetiracetam (LEV) are used [4]. Brivaracetam (BRV) is a racemat derivative with anticonvulsant properties [7, 8]. Literature survey pharmacokinetics performed by analyst software (version: 1.4.2).

Chemicals and reagents

Brivaracetam and brivaracetam D3 (IS) were procured from unichem laboratories Ltd, Mumbai, India, ammonium acetate was procured from merck specialties pvt. ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, MilliPore, USA) installed in the laboratory. The formic acid analytical grade was supplied by J. T. Baker, USA, Hyderabad.

Calibration standard solutions

Stock solutions of brivaracetam and brivaracetam D6 internal standard (IS) were prepared in methanol. Further dilutions were carried out in 70% methanol. Calibration standards of eight concentration levels were prepared freshly by spiking drug-free plasma with brivaracetam stock solution to give the concentrations of 0.16, 0.6, 0.9, 1.5, 3.00, 4.5, 6 and 8μg/ml.

Quality control standards

Lowest quality control standards, median quality control standards and highest quality control standards were prepared by spiking drug-free plasma with brivaracetam to give a solution containing 0.24, 2.8 and 5.5μg/ml respectively. They were stored at -20 °C until the time analyzed.

Chromatographic conditions

Chromatographic separation was performed on a chromatolith C18column (100 mmx4.6 mmx5 μm) with 0.1% formic acid, adjusted to pH 3.2 as mobile phase with a flow rate of 1.0 ml/min. Injection volume was 5μl. Total analysis time of single injection was 4.9 min. Column oven temperature and autosampler temperature was set to 40 °C and 5 °C respectively.
Mass spectrometric conditions

The LC eluent was split (75%), and approximately 0.25 ml/min was introduced and quantification was achieved with MS/MS detection in negative ion mode for the analytes and IS using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with turboion spray interface at 400 °C. The ion spray voltage was set at 5500 V. The source parameters viz., the nebulizer gas, curtain gas, CAD gas were set at 40, 40 and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) for MT and MT-D3 were similar and are-55,-25,-10,-6 V. For brivaracetam and brivaracetam D3 the DP, CE, EP and CXP were 55,-24,-10 and-18 V. A turbo ion spray interface (TIS) operated in negative ionization mode was used for the detection. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of m transitions of m/z 213.0/168.00 for Brivaracetam and m/z 219.10/174.00 for Brivaracetam D6. Quadrupoles Q1 and Q3 were set on the unit resolution.

Study design

Six Male albino Rabbits (weighing about 2.5 kg) procured by vijaya college of Pharmacy which was obtained from the approved vendor. The Rabbits selected for the study was approved by Institutional Ethical committee no: VCP/IAEC/2016-45. The age of the rabbits was 8-12 w and had no medication for two weeks prior to the study. Twelve hours before drug administration, food was withdrawn from the rabbits until 24 hr post-dosing, while, water was available for rabbits throughout the study. The tablets were administered to rabbits using a balling gun. Blood samples (0.6 ml) were withdrawn from the marginal ear vein before dosing (zero time) and at time intervals of 0.15, 0.25, 0.5, 0.75, 1.0, 1.15, 2.25, 2.5, 3, 4, 5, 6, 7, 8, 10, 12hr after administration. For each animal, the total number of blood samples drawn during the study was 17. EDTA disodium salt was used as an anticoagulant. Plasma was separated by centrifugation at 5000 rpm for 10 min and the resulting plasma sample from each blood sample was divided into two aliquots and stored in suitably labelled polypropylene tubes at-20 °C until used. All the plasma samples were analysed under the construction of standard calibration curve of brivaracetam in rabbit’s plasma. The brivaracetam concentrations in the rabbit plasma samples were calculated using the calibration curve, obtained after linear regression of the peak area ratio (brivaracetam/brivaracetam D6) versus the concentration of brivaracetam.

Sample preparation method

To 400 µl of plasma, 50 µl of brivaracetam D6 (1µg/ml) was added and vortexed. The drug was extracted with 3 ml of ethyl acetate followed by centrifugation at 4000 rpm/min on a cooling centrifuge for 15 min at 4 °C. The organic phase was withdrawn and dried using lyophliser. To the residue, 250 µl of mobile phase was added and transfer appropriate volume of samples into pre-labelled Autosampler vials, and inject by using HPLC-ESI-MS/MS.

Pharmacokinetic analysis

Single dosage pharmacokinetic parameters were calculated using PK Solver tool from plasma drug concentration-time data by non-compartmental methods. The maximum plasma concentration (Cmax) and time to maximum plasma concentration (Tmax) were obtained directly from the observed concentration-time profiles. The linear trapezoidal rule was used to estimate the area under the plasma concentration versus time curve (AUC) from 0 to the last measurable concentration (AUC 0-1). The area under the plasma concentration versus time curve from 0 to infinity (AUC 0-∞) was calculated as AUC 0-t+Ke/Ke, where Ct was the last measurable concentration. Ke was the elimination rate constant. The terminal elimination half-life (t1/2) was calculated as 0.693/Ke.

Validation

Specificity

A solution containing 0.16µg/ml was injected on to the column under optimized chromatographic conditions to show the separation of brivaracetam from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of brivaracetam to the internal standard and the best fit line was calculated. Wide range calibration was determined by solutions containing 0.16µg/ml to 8µg/ml.

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against unextracted quality control samples at HQC, MQC and LQC.

Precision and accuracy

Intraday precision and accuracy was determined by analyzing quality control standards (0.24, 2.8and 5.5µg/ml) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control standards (0.24, 2.8and 5.5µg/ml) on each of five different days.

Matrix effect

The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LQC and HQC of brivaracetam were prepared with six different lots of plasma and are injected.

RESULTS AND DISCUSSION

Results of method validation

The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, HQC, MQC and LQC samples are shown in (fig. 1 to 4).
The method developed was validated for linearity, accuracy and precision, and stability as per ICH guidance [14-17]. The results of validating parameters are given below.

**Linearity**

The three calibration curves (Calculated concentration Vs Actual Concentration) were linear over working range of 0.16μg/ml to 8μg/ml with eight-point calibration used for quantification by linear regression (fig 5). The regression equation for the analysis was $Y=0.0053x+0.0018$ with coefficient of correction ($r^2$) = 0.998. The precision (% CV) observed for the calibration curve standards was found to be ≤ 1.96 for brivaracetam (table 1).

**Recovery**

The % mean recovery for Brivaracetam in LQC(0.24 μg/ml), MQC (2.8μg/ml) and HQC(5.5μg/ml) was 95.7%, 109.8% and 106.5% respectively (table 2).

**Intraday and inter-day precision**

The mean intraday and inter-day precision of the method was found to be 0.77 to 3.72% for the quality control samples. This is within the acceptance limits of precision is 15%. The lower limit of Quantification was found to be 0.16μg/ml at such concentration, the mean interday and intraday precision was found to be 0.96% and 3.21% respectively. Which are within the acceptance limits of precision is 20%.(table 3).
Table 1: Linearity standards of brivaracetam

| Actual conc. (μg/ml) | 0.160 | 0.600 | 0.900 | 1.50 | 3.00 | 4.50 | 6.00 | 8.00 | Slope | Intercept |
|---------------------|-------|-------|-------|------|------|------|------|------|-------|------------|
| 1                   | 0.158 | 0.58  | 0.901 | 1.527| 3.016| 4.51 | 6.19 | 7.82 | 0.994 | 0.03       |
| 2                   | 0.155 | 0.58  | 0.909 | 1.499| 3.016| 4.46 | 6.31 | 7.85 | 1.002 | 0.006      |
| 3                   | 0.159 | 0.59  | 0.916 | 1.477| 2.963| 4.43 | 6.14 | 7.76 | 0.985 | 0.017      |
| Mean                | 0.157 | 0.583 | 0.908 | 1.501| 3.02 | 4.46 | 6.23 | 7.81 | 0.993 | 0.017      |
| ±SD                 | 0.002 | 0.005 | 0.007 | 0.025| 0.059| 0.040| 0.087| 0.045| 0.008 | 0.012      |
| %CV***              | 1.32  | 0.99  | 0.83  | 1.67 | 1.96 | 0.90 | 1.41 | 0.59 |       |            |
| LOD****             | 0.099 μg/ml | 0.120 μg/ml |
| LOQ††††             |       |       |       |      |      |      |      |      |       |            |

* Standard deviation, ** coefficient of variation, *** limit of detection, **** limit of quantification.

Fig 5: Spiked concentrations (0.16 μg/ml to 8.0 μg/ml) were plotted against peak area ratio Vs concentration with nine-point calibration used for quantification by linear regression

Table 2: The % mean recovery of brivaracetam for LQC, MQC and HQC

| ID | LQC (0.24 μg/ml) | MQC (2.8μg/ml) | HQC (5.5μg/ml) |
|----|-----------------|----------------|----------------|
|    | Un extracted (area ratio) | Extracted (area ratio) | % Recovery | Un extracted (area ratio) | Extracted (area ratio) | % recovery | Un extracted (area ratio) | Extracted (area ratio) | % Recovery |
| 1  | 0.159           | 0.146          | 91.824        | 0.96          | 1.044          | 108.75     | 2.017          | 2.098          | 104.02     |
| 2  | 0.156           | 0.141          | 90.385        | 0.974         | 1.052          | 108.01     | 2.057          | 2.1           | 110.09     |
| 3  | 0.142           | 0.139          | 97.887        | 0.981         | 1.037          | 105.71     | 1.854          | 2.084          | 112.41     |
| 4  | 0.152           | 0.148          | 97.368        | 0.926         | 1.04           | 112.31     | 1.988          | 2.091          | 105.18     |
| 5  | 0.141           | 0.134          | 99.291        | 0.931         | 1.045          | 112.24     | 1.929          | 2.036          | 105.55     |
| 6  | 0.158           | 0.154          | 97.468        | 0.936         | 1.049          | 112.07     | 1.913          | 2.106          | 110.09     |
| Mean | 0.151         | 0.145          | 95.704        | 0.951         | 1.045          | 109.849    | 1.960          | 2.086          | 106.55     |
| ±SD* | 0.008         | 0.006          | 3.657         | 0.023         | 0.066         | 2.774      | 0.075          | 0.026          | 3.899      |
| %CV** | 5.28           | 4.00           | 3.82          | 2.47          | 0.53          | 2.53       | 3.81           | 1.23           | 3.66       |

* Standard deviation, ** coefficient of variation.

Table 3: Intra-day and inter-day quality control samples for brivaracetam

| Intra-batch | Brivaracetam (ng/ml) | LLOQ QC (0.16 μg/ml) | LQC (0.24 μg/ml) | MQC (2.8μg/ml) | HQC (5.5μg/ml) |
|-------------|----------------------|----------------------|-----------------|----------------|----------------|
| Mean°       | 0.151                | 0.254                | 2.741           | 5.669          |                |
| SD°°        | 0.004                | 0.003                | 0.056           | 0.044          |                |
| %CV°*       | 3.21                 | 3.22                 | 2.06            | 0.77           |                |
| Mean        | 0.153                | 0.219                | 2.811           | 5.823          |                |
| SD          | 0.0015               | 0.008                | 0.056           | 0.068          |                |
| %CV         | 0.96                 | 3.72                 | 2.761           | 5.692          |                |
| Mean        | 0.156                | 0.244                | 2.761           | 5.692          |                |
| SD          | 0.0021               | 0.009                | 0.052           | 0.085          |                |
| %CV         | 1.39                 | 3.64                 | 1.9             | 1.49           |                |
| Inter-batch | LLOQ QC (0.16 μg/ml) | LQC (0.24 μg/ml) | MQC (2.8μg/ml) | HQC (5.5μg/ml) |
| Mean        | 0.155                | 0.254                | 2.657           | 5.469          |                |
| SD          | 0.002                | 0.003                | 0.081           | 0.081          |                |
| %CV         | 1.8                  | 3.22                 | 3.06            | 1.47           |                |

° Average of six determinations, °° standard deviation, °°° Coefficient of variation.
Table 4: Matrix effect obtained with six different lots of plasma

| QC ID | LQC | HQC |
|-------|-----|-----|
| Actual conc. (µg/ml) | 0.24 | 5.5 |
| 1    | 0.266 | 6.125 |
| 2    | 0.263 | 6.089 |
| 3    | 0.252 | 6.033 |
| 4    | 0.26  | 6.048 |
| 5    | 0.277 | 5.865 |
| 6    | 0.255 | 6.313 |
| Mean | 0.262 | 6.079 |
| ±SD* | 0.009 | 0.145 |
| % CV** | 3.39 | 2.39 |

*Standard deviation, **coefficient of variation.

Table 5: Calculated plasma concentrations in rabbits at each time point

| Time points (h) | Rabbit 1 | Rabbit 2 | Rabbit 3 | Rabbit 4 | Rabbit 5 | Rabbit 6 | Mean | SD* |
|----------------|----------|----------|----------|----------|----------|----------|------|-----|
| 0              | 0        | 0        | 0        | 0        | 0        | 0        | 0    | 0   |
| 0.15           | 18       | 20       | 21       | 14       | 17       | 20       | 18.33| 2.58|
| 0.25           | 36       | 31       | 34       | 33       | 39       | 36       | 34.83| 2.79|
| 0.5            | 54       | 60       | 62       | 60       | 54       | 58       | 58.00| 3.35|
| 0.75           | 73       | 83       | 81       | 71       | 67       | 71       | 74.33| 6.28|
| 1              | 86       | 98       | 93       | 89       | 95       | 91       | 92.00| 4.29|
| 1.15           | 79       | 85       | 91       | 84       | 90       | 95       | 87.33| 5.75|
| 2.25           | 70       | 77       | 78       | 72       | 78       | 83       | 76.33| 4.68|
| 2.5            | 65       | 60       | 75       | 68       | 71       | 80       | 69.83| 7.14|
| 3              | 60       | 56       | 66       | 64       | 64       | 69       | 63.17| 4.58|
| 4              | 57       | 63       | 63       | 59       | 56       | 51       | 58.17| 4.58|
| 5              | 54       | 58       | 59       | 51       | 49       | 43       | 52.33| 5.99|
| 6              | 51       | 55       | 52       | 44       | 38       | 37       | 46.17| 7.63|
| 7              | 48       | 51       | 47       | 42       | 35       | 34       | 42.83| 7.08|
| 8              | 33       | 40       | 40       | 33       | 31       | 35       | 35.33| 3.83|
| 10             | 15       | 2        | 2        | 2        | 2        | 2        | 2    | 5.31|
| 12             | 0        | 0        | 0        | 0        | 0        | 0        | 0    | 0   |

*Standard deviation.

Fig. 6: Plasma time profile curves of test animals plotted between sampling time points (17) and mean (n=6) plasma concentrations

Table 6: Calculated mean values of pharmacokinetic parameters for test animals

| Parameter            | Rabbit 1 | Rabbit 2 | Rabbit 3 | Rabbit 4 | Rabbit 5 | Rabbit 6 | Mean | SD* |
|----------------------|----------|----------|----------|----------|----------|----------|------|-----|
| Lambda_z             | 0.308647 | 1.13318 | 1.15981 | 1.07014  | 1.01354  | 1.01392  | 0.95692| 0.283|
| t1/2                 | 1.783487 | 0.608388 | 0.62111 | 0.647738 | 0.63883  | 0.63862  | 0.83808| 0.464|
| Tmax                 | 1        | 1        | 1        | 1        | 1        | 1        | 1.15  | 0.061|
| Cmax                 | 86       | 98       | 93       | 89       | 95       | 95       | 92.6667| 4.412|
| Tlag                 | 0        | 0        | 0        | 0        | 0        | 0        | 0     | 0.000|
| Clast_obs/Cmax       | 0.174419 | 0.020408 | 0.021505 | 0.022472 | 0.02105  | 0.02105  | 0.04681| 0.063|
| AUC0-t               | 498      | 521.875  | 533.075  | 480.175  | 468.225  | 475.9    | 496.208| 26.358|
| AUC0-inf_obs        | 536.5954 | 523.6304 | 534.8671 | 482.044  | 470.1983 | 477.8725 | 504.2013| 30.684|
| AUC0-0-t/0-inf_obs  | 0.928074 | 0.996648 | 0.996649 | 0.996123 | 0.995803 | 0.995872 | 0.984861| 0.028|
| AUC0-inf_obs        | 256.9442 | 215.776  | 215.627  | 190.333  | 178.73   | 180.971  | 206.2529| 29.455|
| MRT 0-inf_obs       | 4.789162 | 4.111251 | 4.026472 | 3.9482   | 3.80633  | 3.779199 | 4.076776| 0.371|
| Vz/F_obs            | 0.47951  | 0.167622 | 0.167532 | 0.19386  | 0.209834 | 0.206385 | 0.237457| 0.120|
| CI/F_obs            | 0.18636  | 0.199974 | 0.186962 | 0.20745  | 0.212676 | 0.209261 | 0.198947| 0.012|

*Average of six determinations, **standard deviation.
Matrix effect
The % CV for HQC and LQC samples was observed 2.39% and 3.39% respectively (table 4), which are within 15% as per the acceptance criteria.

Results of pharmacokinetic studies
The Pharmacokinetic parameter of brivaracetam was calculated from the plasma concentration-time curves using pk solver software. Also, the area under the plasma concentration-time curve from 0 to 12 hr (AUC0-12) was calculated using trapezoidal rule. Brivaracetam showed Tmax of 1.02±0.061 and mean Cmax, AUC0→t and AUC0→∞ for Test formulation is 92.7±4.4, 496.21±26.4 and 504.20±30.68 respectively the results were presented in table 5, table 6 and fig. 6.

DISCUSSION
The established LC-MS/MS method was linear with least LLOQ (0.16μg/ml) concentration and have a good recovery when compared to other reported method for the estimation of brivaracetam metabolites from human plasma and another biological matrix. [9-13]. As per literature review, no LC-MS/MS method was available for determination of brivaracetam alone from rabbit plasma, the validated method was successfully applied for the determination of Tmax, Cmax, AUC0→t and AUC0→∞ using rabbits as test animals. The pharmacokinetic parameters were evaluated through linear trapezoidal rule and results found were most promising. Hence the developed method can be applied for bioanalytical and bioequivalence studies of brivaracetam.

CONCLUSION
The bio-analytical methodology for determination of brivaracetam described in this manuscript is highly specific, rugged and rapid for therapeutic drug monitoring both for analysis of routine samples of a single dose or multiple dose pharmacokinetics and also for clinical trial samples with desired sensitivity, precision, accuracy and high throughput. The method involved a simple and specific sample preparation by liquid-liquid extraction followed by isocratic chromatographic separation in 2.0 min. The overall analysis time is promising compared to other reported procedures for brivaracetam. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of brivaracetam.

AUTHOR CONTRIBUTION
The corresponding author Mr. Darshan Bhatt, Research Scholar, Mewar University, Chittorgarh, Rajasthan, India, who completed the intended research work under the guidance of his guide Dr. B. Rajkamal, Research Supervisor, Mewar University, Chittorgarh, Rajasthan, India.

CONFLICT OF INTERESTS
Declared none

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