

Original Article

A VALIDATED LC-MS/MS METHOD FOR PHARMACOKINETIC STUDY OF BRIVARACETAM IN HEALTHY RABBITS

D. ATUL VASANTH*, B. RAJKAMAL

Mewar University, Chittorgarh, Rajasthan, India
Email: atulvasanth99@gmail.com

Received: 19 Jul 2017 Revised and Accepted: 21 Dec 2017

ABSTRACT

Objective: A liquid chromatography-tandem mass spectrophotometric (LC-MS/MS) method was developed for quantification of brivaracetam in rabbit plasma employing liquid-liquid extraction with ethyl acetate.

Methods: Developed method was validated for specificity, precision, accuracy, recovery, and stability characteristics. Chromatographic separation was achieved on Chromolith C18 column (100 mmx4.6 mmx5 μ m) with 0.1% formic acid, adjusted to pH 3.2 as an isocratic mobile phase with a flow rate of 1.0 ml/min. The developed method was applied to assess pharmacokinetics parameters like C_{max} , T_{max} , $t_{1/2}$ and AUC of brivaracetam in healthy rabbits.

Results: The developed method was linear over the range of 0.16 to 8 μ g/ml. The regression equation for the analysis was $Y=0.0053x+0.0018$ with coefficient of correlation (r^2) = 0.998. The % mean recovery for brivaracetam was found to be between 95.7% to 106.5%. The mean intraday and inter-day precision of the method was found to be 0.77 to 3.72% for quality control standards. Brivaracetam showed T_{max} of 1.025 ± 0.061 and mean C_{max} , $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ for Test formulation is 92.7 ± 4.4 , 496.21 ± 26.4 and 504.20 ± 30.68 respectively.

Conclusion: A highly specific, rugged and rapid method with sufficiently low LLOQ was developed for analysis of routine samples of a single dose or multiple dose pharmacokinetic studies with any marketing formulation of brivaracetam.

Keywords: Brivaracetam, Pharmacokinetics, LC-MS/MS, Rabbit model

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DOI: <http://dx.doi.org/10.22159/ijpps.2018v10i2.21457>

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 are the preferred first-line treatment of acute seizures and SE [2]. If seizures are not fully controlled with benzodiazepines, second-line AEDs such as phenytoin/fosphenytoin, sodium valproate, lacosamide, [3] or the SV2A AED levetiracetam (LEV) are used [4]. Brivaracetam (BRV) is an analog of LEV, rationally designed to have a higher affinity and selectivity for SV2A, a protein involved in synaptic vesicle exocytosis and neurotransmitter release [5, 6]. Brivaracetam chemically is a 4-n-propyl analog of levetiracetam and a racetam derivative with anticonvulsant properties [7, 8]. Literature survey pharmacokinetics and metabolism of ¹⁴C-brivaracetam, metabolism studies of brivaracetam and gemfibrozil, clinical trials of adjunctive brivaracetam for refractory partial-onset seizures, identification of drug metabolites in human plasma or serum integrating metabolite prediction, by LC-HRMS methods are reported for the drug [9-13]. To best of our knowledge, no published LC-MS/MS-based methods for the pharmacokinetic study of brivaracetam in healthy rabbits. Therefore a liquid chromatography-tandem mass spectrophotometric (LC-MS/MS) method was developed, validated and applied for quantification of brivaracetam in rabbit plasma employing liquid-liquid extraction (LLE) technique. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of brivaracetam in human volunteers.

MATERIALS AND METHODS

Apparatus and software

The HPLC system with an autosampler was a Shimadzu LC-20ADvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex,

Canada) API 4000 tandem mass spectrometer. The autosampler was SIL-HTC from Shimadzu, Japan. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was 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, Milli Pore, 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 8 μ g/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 5.5 μ g/ml respectively. They were stored at -20 °C till the time analyzed.

Chromatographic conditions

Chromatographic separation was performed on a Chromolith C18 column (100 mmx4.6 mmx5 μ m) 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 5 μ l. 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 quantitation 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 lyophiliser. 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 (C_{max}) and time to maximum plasma concentration (T_{max}) 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-t). The area under the plasma concentration versus time curve from 0 to infinity (AUC 0-∞) was calculated as AUC 0-t+C_t/k_e, where C_t was the last measurable concentration. K_e was the elimination rate constant. The terminal elimination half-life (t_{1/2}) was calculated as 0.693/K_e.

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) and LLOQ quality control standard (0.16µg/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) and LLOQ quality control standards (0.16µg/ml) once 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).

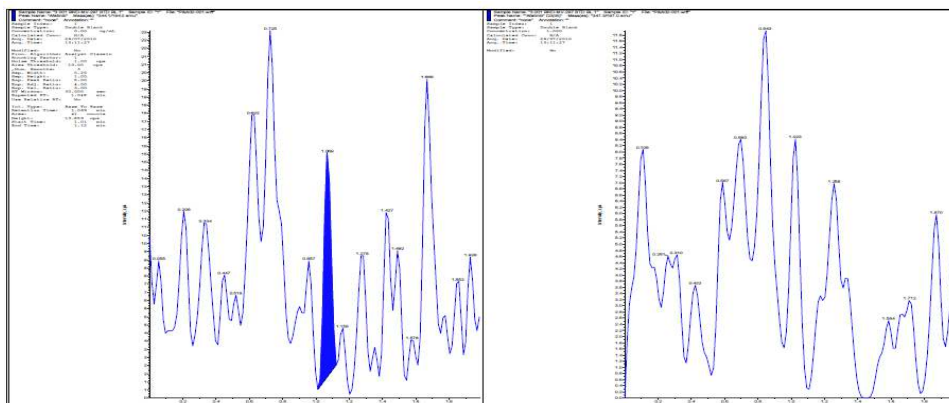


Fig. 1: Representative blank chromatograms of brivaracetam and IS in blank plasma

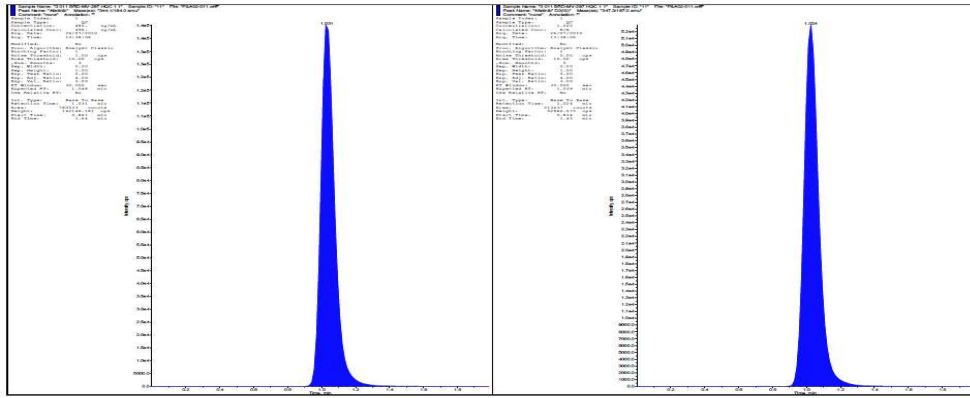


Fig. 2: Representative HQC-chromatograms of brivaracetam in plasma with internal standard

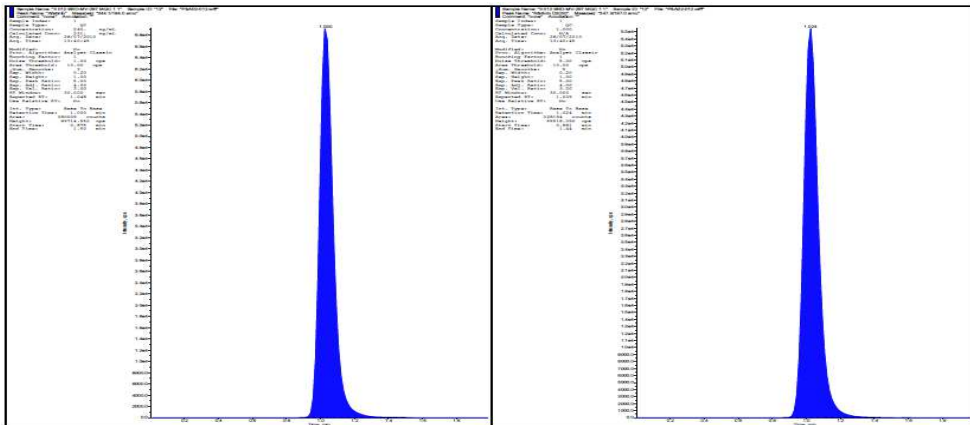


Fig. 3: Representative MQC-chromatograms of brivaracetam and its internal standard

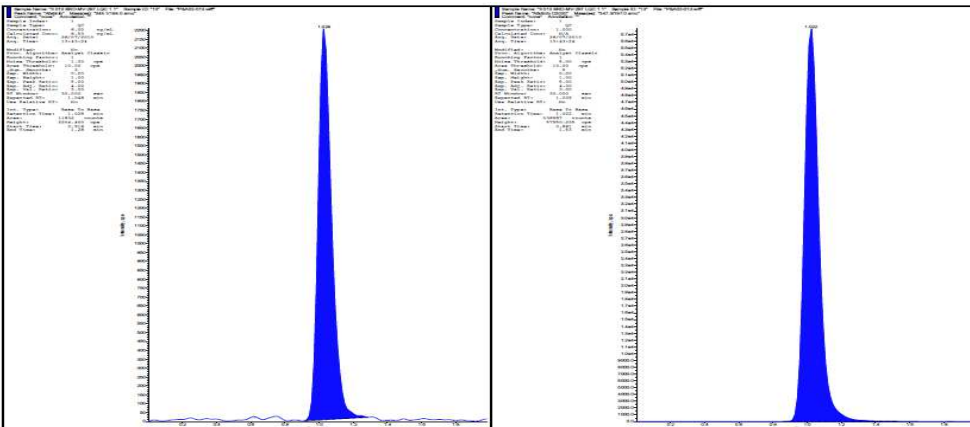


Fig. 4: Representative chromatograms of brivaracetam and its internal standard at LQC Level

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 0.16 μ g/ml to 8 μ g/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 μ g/ml), MQC (2.8 μ g/ml) and HQC(5.5 μ g/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 0.16 μ g/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	3.081	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	4.466	6.213	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***	0.039 µg/ml									
LOQ****	0.120 µg/ml									

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

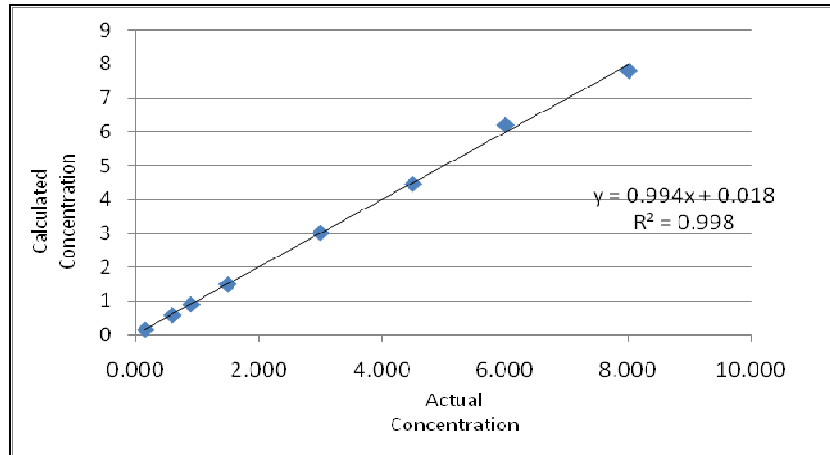


Fig. 5: Spiked concentrations (0.16 µg/ml to 8.0 µg/ml) were plotted against peak area ratio Vs concentration with nine-point calibration used for quantification by linear regression

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	102.09
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	0.14	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	0.006	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	1.16
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

Calculated concentrations (µg/ml)								
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	4.17	5.31
12	0	0	0	0	0	0	0	0

*Standard deviation.

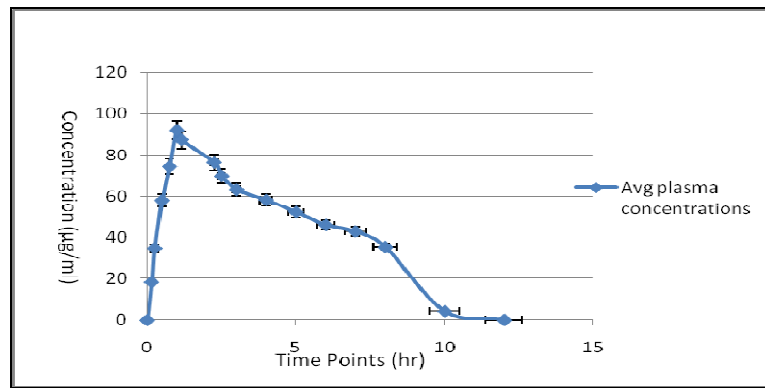


Fig. 6: Plasma time profile curves of test animals plotted between sampling time points (17) and mean (n=6) plasma concentrations

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	0.388647	1.139318	1.115981	1.070104	1.013546	1.013932	0.956921	0.283
t1/2	1.783487	0.608388	0.621111	0.647738	0.683883	0.683623	0.838038	0.464
Tmax	1	1	1	1	1	1.15	1.025	0.061
Cmax	86	98	93	89	95	95	92.66667	4.412
Tlag	0	0	0	0	0	0	0	0.000
Clast_obs/Cmax	0.174419	0.020408	0.021505	0.022472	0.021053	0.021053	0.046818	0.063
AUC 0-t	498	521.875	533.075	480.175	468.225	475.9	496.2083	26.358
AUC 0-inf_obs	536.5954	523.6304	534.8671	482.044	470.1983	477.8725	504.2013	30.684
AUC 0-t/0-inf_obs	0.928074	0.996648	0.996649	0.996123	0.995803	0.995872	0.984861	0.028
AUMC 0-inf_obs	2569.842	2152.776	2153.627	1903.23	1789.73	1805.971	2062.529	296.455
MRT 0-inf_obs	4.789162	4.111251	4.026472	3.94825	3.80633	3.779189	4.076776	0.371
Vz/F_obs	0.47951	0.167622	0.167532	0.19386	0.209834	0.206385	0.237457	0.120
Cl/F_obs	0.18636	0.190974	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 T_{max} of 1.025±0.061 and mean C_{max}, AUC_{0→t} and AUC_{0→∞} 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 T_{max}, C_{max}, AUC_{0→t} and AUC_{0→∞} 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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