Method Development and Validation of a Novel UHPLC Coupled with MS/MS Method for the Estimation of Brivaracetam in Human (K_2EDTA) Plasma Samples and its Application to Pharmacokinetic Study

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Running Title: A Novel UHPLC Coupled with MS/MS Method for the Estimation of Brivaracetam

ABSTRACT

Background: Brivaracetam is a novel antiepileptic drug, approved clinically for the treatment of partial onset seizures in adults and adolescents. It has some abuse potential and assigned to Schedule V category under the Controlled Substance Act by the Drug Enforcement Administration. It is essential to develop a faster, simple and highly sensitive method for the quantification of Brivaracetam in human plasma by employing simple liquid-liquid extraction.

Objective: To develop and validate a novel UHPLC-MS/MS method for the estimation of brivaracetam in human plasma samples and application to pharmacokinetic study.

Methods: An ultra high-pressure liquid chromatography-tandem mass spectrometry method was developed and validated according to current regulatory guidelines for bioanalytical methods. Sample processing (50 µL) involved only a simple liquid-liquid extraction by ethyl acetate as extraction solvent. Brivaracetam-d7 was used as internal standard. The chromatographic analysis was performed by a Unisol C18 (4.6 X 100 mm, 5µm) column using 0.1% formic acid in water/acetonitrile (20/80 V/V) as an isocratic mobile phase, at a flow rate of 1.0 mL/min with a run time of 2.2 min. Brivaracetam and its internal standard Brivaracetam D7 were detected and quantified in positive ion mode using multiple reaction monitoring transitions at m/z 213.100→168.100 and m/z 220.000→175.100 respectively. The developed method was applied to assess pharmacokinetic parameters like Cmax, Tmax, t1/2 and AUC for Brivaracetam in healthy, male, adult humans.

Results: The method was validated over a concentration range of 20.000 ng/mL to 4000. 000 ng/mL. Both intra- and inter-assay imprecision and inaccuracy were <15% for all quality control samples. No matrix effect was observed. Pharmacokinetic results showed that test formulation is bioequivalent with reference formulation.

Conclusion: The present assay is faster, highly sensitive and simpler than previously published analytical reports for brivaracetam in human plasma samples and is suitable for pharmacokinetic evaluation of any marketed formulation.

Keywords:

Brivaracetam, Liquid-Liquid Extraction (LLE), UHPLC, MS/MS, Bioanalytical Method Validation, Pharmacokinetics.

1. INTRODUCTION

Brivaracetam (BRV, Figure 1) is a new antiepileptic drugs used to treat seizures (partial onset) in adults [1, 2]. United States Food and Drug Administration (USFDA) has approved BRV in 2016 for adjunctive treatment for focal seizures in patients aged ≥16 years with epilepsy (FDA 2016). BRV is a selective, high affinity ligand for synaptic vesicle 2A (SV2A) is a transmembrane glycoprotein and galactose transporter [3, 4]. The drug has higher affinity than Levetiracetam (15 to 30 fold higher). BRV was well tolerated. It shows favourable pharmacokinetic profile in a wide dose range i.e., single oral dose (10 mg to 1000 mg) also multiple oral doses (200 mg/day to 800 mg/day) in phase studies [5, 6]. Following oral administration (single or multiple doses), peak plasma concentrations of BRV was attained at 1h (t_{max}) [7, 8, 1]. The studies indicate there was a food effect on pharmacokinetic BRV. High fat food decreases the concentration maxima (C_{max}) and delays t_{max} (3 hours). However, on area under the curve for plasma concentration versus time curve (AUC) no effect was there [1, 8]. The drug has very minimal plasma binding (<20%) [9].

As per the available literatures, a very few LC-MS/MS based method was available for estimating BRV in biological sample [8, 10-14]. Of the reported methods available, only three methodologies were comparable with this present work. Iqbal et al., 2017 [10] reported on UPLC-MS based methodology for identifying and quantifying BRV in human plasma. The methodology utilized Carbamazepine as internal standard, and methyl-tert-butyl ether was used as extraction solvent to extract BRV and the ISTD from plasma. The methodology has a run time of 4 minutes. The methodology was satisfactorily utilized in BRV pharmacokinetic study (in rats). Vasanth et al., 2018 [11] describes a methodology for estimating BRV in rabbit plasma samples. A concentration of 160 ng per mL was kept as LLOQ. The above reported method (Vasanth et al., 2018 [11]) was also satisfactorily utilized to derive BRV pharmacokinetics in rabbits. Later, Susan et al., 2020 [14] reported UHPLC-MS/MS assay for the therapeutic monitoring of brivaracetam plasma concentrations in patients with epilepsy. The method was not sensitive and validated over a concentration range of 0.10-10 mcg/mL. Also suitable for therapeutic drug monitoring not for pharmacokinetic application. These methods were suffering from lack of sensitivity [11, 14], use of more sample volume [11], longer chromatographic run time [10] and employs non-deuterated compounds as internal standards [10] which may results poor precision and accuracy values, where compensation for matrix effect is not possible. An efficient bioanalytical method should rapid, simple and consume less sample volume for analysis. Also, it should be specific and selective to avoid possible inferences as mass transition of analyte and ISTD.

Herein, the authors have put forward a simple, easy, fast(rapid) and highly precise UHPLC-MS/MS methodology for quantifying BRV in human plasma. The present methodology utilized limited sample volume of 50µL and partitioning liquid extraction (Liquid-liquid extraction) employing Ethyl acetate for extraction. The method also utilized Brivaracetam-D_7 (Figure 1) as an ISTD to eliminate matrix effect related issues and to minimize the recovery variation between Brivaracetam and ISTD. The above methodology has been satisfactorily utilized in human pharmacokinetic study.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The reference analytical standards for Brivaracetam (97%) and Brivaracetam-d_7 (97%) were procured from BioOrganics Labs Limited (Bengaluru, India). Acetonitrile and Methanol (LC-MS grade) were procured from Scharlau Chemicals (USA). Formic acid (Analytical grade) was procured from Rankem Ltd. (Mumbai, India). Milli-Q (water purification system) (In-house) was used to prepare LCMS grade water for analysis. Human (K_2EDTA) plasma was obtained from Deccan Pathological Labs (Hyderabad, India).

2.2 Instruments used, Optimized Conditions

A Shimadzu Nexera X2 (Kyoto, Japan) UHPLC System coupled with an SCIEX Triple Quad 4500 Mass Spectrometer (Sciex, Foster City, CA, USA) with TurboV ionspray^{TM} (ESI) ionisation source was utilized for the study. The optimized chromatographic and Mass spectrometric parameters/conditions were listed in Table 1.

2.3 Stock and Working Solution Preparation

LC-MS grade Methanol was utilized for preparing the stock solutions of BRV and ISTD and a mixture of Milli-Q/HPLC grade Water : Methanol (50:50, v/v) was used for additional further stock dilutions. Ten-point calibration curve (CC) was prepared in plasma at a concentration of 20.0, 40.0, 80.0, 200.0, 400.0, 800.0, 1600.0, 2400.0, 3200.0 and 4000.0 ng per mL. Quality control (QC) samples preparation was done in plasma at a concentration of 20. 0 ng per mL (LLOQQC). 60. 0 ng per mL (low QC - LQC), 1800. 0 ng per mL (middle QC - MQC) and 3000.0 ng per mL (high QC - HQC). Spiked Calibration Standards and Quality Control samples storage was executed in a deep-freezer maintained at -70±10°C until use. The CC and QC samples were prepared from independent stock solutions. Milli-Q/HPLC grade Water : Methanol (50:50, v/v) was utilized for preparing ISTD stock dilution (200. 0 ng/mL). Storage of the stock solutions and stock dilutions was done at -10°C during their usage.

2.4 Sample Processing/Preparation

Prepared samples were taken out from deep-freezer and thawed in a water bath kept at room temperature. It was vortex mixed to completely homogenise the content. Into the labelled polypropylene tube, then added, 50 µL aliquot of IS dilution (200 ng/mL), 50 µL of plasma (spiked) sample and 50 µL 0.1% formic acid (extraction additive) and vortex mixed. Then, added 1mL of Ethyl acetate and vortex mixed for approximately 5 minutes at 2500 rpm. Centrifuged at 4000 rpm, at 5°C for approximately 5 minutes. Collected an aliquot of 0.8 mL of supernatant into glass tubes and dry evaporated over a nitrogen gas stream at 40°C. Added 0.500 mL mobile phase, to the dry evaporated tubes, vortex mixed to reconstitute and injected onto the LC-MS/MS instrument.

2.5 Parameters of Method Validation

Method Validation was performed for the developed method following the recent USFDA - Bioanalytical Method Validation guidelines [15]. This methodology was tested for carryover, selectivity, specificity, sensitivity, accuracy and precision, recovery, sample dilution and stabilities.

2.6 Design of the Pharmacokinetic Study

A Pharmacokinetic study of BRV in healthy male subjects (n=14) was conducted. A protocol was designed before initiating the study and independent ethics committee approved it. Subsequent to oral administration of BRV (100 mg), at pre-dose and 0. 25, 0. 50, 0. 75, 1. 00, 1.25, 1. 50, 1.75, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.50, 5. 00, 6.00, 8.00, 10.00, 12.00, 24. 00 and 48. 00**,** collected the blood samples in vacutainer blood sample collection tubes (BD, Franklin, NJ, USA) containing K_2EDTA under fed condition. Centrifuged these tubes at an rpm (rotation per minute) of 3500 for 10 minutes to separate supernatant plasma. It was followed by collection of the separated plasma and stored the samples at -70±10°C. Analysis of the samples was performed as per the procedure mentioned above. Phoenix WinNonlin version 8.1 was employed for the calculation of Pharmacokinetic parameters of BRV.

3. RESULTS AND DISCUSSION

3.1 Method Development

For developing a simple, easy, fast(rapid) also, extremely precise UHPLC-MS/MS method for estimating BRV in human (K_2EDTA) plasma, applicable for analysing samples of bioavailability/bioequivalence and pharmacokinetic studies was the objective of the study. To attain this, various possibilities were assessed including optimisation of MS parameters/conditions, sample extraction and chromatography. Sciex Triple QUAD 4500 Mass spectrometric conditions were optimized using flow injection method. Prepared the infusion solution of Brivaracetam and ISTD (Internal Standard) having 100.0 ng per mL concentration and infused into mass spectrometer source with 5µL/minute as flow rate. MS Ion-source parameters i.e., curtain-gas, ion spray voltage and Ion-source temperature were monitored to achieve high intensity and stable precursor and product ion for the analyte and the IS. Similarly, collision energy and collision gas were carefully evaluated to obtain high intensity and stable product ion for the Q3 MS ion of the analyte and ISTD (Internal Standard). The maximum intense mass transition was obtained for m/z 213.1 paired with 168.1 for BRV and m/z 220.0 paired with 175.1 for ISTD. The product ion MS spectra of BRV and ISTD were presented in the Figure 2A & 2B. Multiple Reaction Monitoring (MRM) was utilized for estimating BRV.

For LC-MS analysis, Methanol and Acetonitrile were commonly employed as organic solvents. Similarly, salts such as ammonium acetate and ammonium formate, volatile acids e.g. acetic acid, formic acid were used in the analysis. Initially, Acetonitrile and Methanol were evaluated in combination with ammonium acetate and ammonium formate. We also checked the effect of formic

acid as mobile phase acidifier. The use of minimal content (0.05 %) of formic acid as mobile phase acidifier helped, achieving high area response with excellent chromatographic peak symmetry and consistent response of the analyte. Also, a variety of C_{18} based columns (Unisol C_{18}, Zodiac C_{18}, Zorbax SB C_{18}, Hypersil BDS, Zorbax SB C_{18}, Altima HP C_{18}, Ace 5 C_{18} etc) columns were tested to get better chromatography with short retention time for analyte. Among different mobile phase compositions evaluated, Acetonitrile : 0.05% Formic Acid solution in Milli-Q/HPLC grade water (85: 15 v/v) gave symmetric as well as excellent chromatographic peak shape and reproducible analyte response. Best chromatography results were obtained with Unisol C18 (100 mm X 4. 6 mm, 5µm). The run time was kept at approximately 2.2 minutes having an RT (Retention Time) approximately 1.15 minutes for BRV and ISTD.

Clean samples are needed for better MS performance and quality of analysis. Improper sample clean up may result in extracting the interfering endogenous components (from the matrix) in extracted samples and it may decrease the method performance in terms of accuracy and reproducibility. Partitioning Liquid Extraction (Liquid-Liquid Extraction LLE) is mostly preferred extraction technique. It can provide good extraction efficiency (recovery) and also can eliminate or minimize matrix effect. Hence, Partitioning Liquid Extraction (Liquid-Liquid Extraction LLE) was evaluated employing tertiary butyl methyl ether, Ethyl Acetate, Hexane and Diethyl Ether. Of these solvents tested, highest as well as reproducible recoveries were obtained with methyl-tert-butyl ether, also with, Ethyl acetate as reported by the other authors [10, 11] and ethyl acetate was used as extraction solvent in the present study. Isotope-labelled compounds are of choice as ISTD for LC-MS/MS analysis as they increase the assay precision and accuracy [16, 17]. Hence, stable labelled isotope Brivaracetam-D7 was used as ISTD, to quantitate BRV in human (K_2EDTA) plasma samples for the present study.

3.2 Assay Methodology Performance

Carryover test was conducted to ensure no injector carryover in the subsequent injections. An extracted blank sample was injected immediately after ULOQ with ISTD sample to check the carryover impact on the subsequent runs. The method showed zero percent carryover for the analyte and the IS blank sample. Method Selectivity was tested in 6 lots of plasma (4 normal, 1 lipemic and 1 haemolyzed) obtained from different individuals. No significant interference was observed, in all the blank plasma lots at RT of BRV and ISTD (Figure 3). Besides, deuterated ISTD (Brivaracetam-d_7) was contributing no significant interference at the RT of BRV (Figure 4). The method showed good sensitivity with a S/N ratio ≥10 at LLOQ level (Figure 5). LLE with Ethyl Acetate showed negligible matrix effect (calculated as MF-Matrix Factor) determined at LQC and HQC quality control levels evaluated as ISTD Normalised Matrix Factor. Comparison was made between the area of post-extracted samples against mean area of aqueous samples (neat/in-solvent comparison samples). The ISTD Normalized Matrix Factor (%CV) was 1.011 (1.09 %) for LQC and 1.010 (0.50%) for HQC. The extraction recovery for BRV were found to be 79.09%, 87.10% and 85.69% at low, middle and high quality control levels respectively with recovery (mean) of 83.94±4.31%. The recovery of ISTD was 80.79% with the precision of 1.01-13.74%.

The calibration standards curve of BRV, ranging from 20 ng per mL to 4000 ng per mL (having 10 standards points) was linear, using weighing factor of $1/x^2$. Comparison was made between two weighing factors for regression calculation (1/x and $1/x^2$), during method validation, to evaluate the concentration versus detector response. It was observed that regression equation (correlation coefficient) when using the weighing factor of $1/x^2$ was found to be the best fit. Hence, $1/x^2$ weighing model was used for the study. During the method validation, the mean correlation coefficient was ≥0.99. A total of 3 accuracy and precision batches were run in 2 different days. Each batch contains Blank sample, Blank+ISTD sample, Calibration Standards and 6 sets of QCs at each level. Result of Intra-day and inter-day accuracy and precision run of BRV is summarized in Table 2. Sample dilution experiment was performed by diluting the QC sample (1.6 time of ULOQ, 6400 ng/mL) with screened blank plasma. Two-fold and four-fold dilution was performed during the validation. The accuracy and precision (%CV) for 2 times dilution was 98.58% and 3.47% respectively. Similarly, the accuracy and precision (%CV) for 4 times dilution was 99.81% and 10.46% respectively.

Stock solution stability of BRV and the IS was established for 35 days at -10°C. The % stability (with % CV range) for BRV and for ISTD was 98.96 % (2.38-4.18%) and 102.81% (1.36-2.20%), respectively. The stock solutions were deemed to be stable if the deviation is within ±10%. Stability stock solutions were compared with freshly prepared stock solutions. Similarly, whole blood stability was acceptable for BRV at room- temperature/bench-top for 2 hours and the percentage stability from comparison samples was 104.19% and 99.16% at LOQ and HQC levels, respectively.

In addition to the above stability, bench-top stability for 8h, freeze-thaw stability 5 cycles, and processed samples stability (wet extract stability 42 h and autosampler stability 30 h) were also evaluated at Low QC - LQC and High QC - HQC levels (n=6). Similarly, long-term stability (-70±10°C for 70 days) in spiked samples (in plasma) was evaluated at LQC and HQC levels. Freshly prepared calibration curve standards were used for the stability evaluation. The samples were considered stable when the % accuracy or % stability is ±15% from the nominal value and the precision should be ≤15% (Table 3). No deviation was observed the validation.

3.3 Results of the Pharmacokinetic Study

Above validated methodology was employed for estimating BRV plasma concentration during the pharmacokinetic study in male healthy adult volunteers/subjects (n=14) Figure 6 represents the mean plasma concentration versus time profile for BRV subsequent to oral administration of BRV tablet under fasting condition. The relevant pharmacokinetic parameters were tabulated in Table 4.

4. CONCLUSIONS

In supposition, we successfully developed and validated a novel, simple, fast also of extreme sensitivity UHPLC-MS-MS method of estimating BRV in human (K_2EDTA) plasma. Isotope labelled compound was employed as ISTD (Internal Standard) to obtain accurate as well as reproducible results. The simple extraction procedure with Ethyl Acetate gave a high recovery for Brivaracetam and ISTD (Internal

Standard). The analyte was stable at the various conditions of stability evaluated. The methodology was fast with approximate run time of 2. 2 minutes per sample, thus increasing the number of samples whose analysis can be done in a day. The methodology was discovered to be applicable to pharmacokinetic study in humans, further this methodology can be valuable for bioavailability and bioequivalence in generic drug development process.

5. LIST OF ABBREVIATIONS

BRV	:	Brivaracetam
HQC	:	High Quality Control
ISTD	:	Internal Standard
K_2EDTA	:	Dipotassium Ethylene Diamine Tetra Acetic acid
LQC	:	Low Quality Control
LLOQ	:	Lower Limit of Quantification
LLE	:	Liquid Liquid Extraction
MS/MS	:	Mass Spectrometry/ Mass Spectrometry
MQC	:	Middle Quality Control
RT	:	Retention Time
ULOQ	:	Upper Limit Of Quantification
UHPLC	:	Ultra High Pressure Liquid Chromatography

ETHICS APPROVAL AND CONSENT TO PARTICIPATION

All the experiments were conducted as per ethical standards or Helsinki declaration.

HUMAN AND ANIMAL RIGHTS

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

[1] Klein, P.; Diaz, A; Gasalla, T.; Whitesides, J. A review of the pharmacology and clinical efficacy of brivaracetam. *Clin. Pharmacol.,* **2018**, 10, 1-22.

[2] Klitgaard, H.; Matagne, A.; Nicolas, J.M.; Gillard, M.; Lamberty, Y.; De Ryck, M.; Kaminski, R.M.; Leclercq, K.; Niespodziany, I.; Wolff, C.; Wood, M.; Hannestad, J.; Kervyn, S.; Kenda, B. Brivaracetam:Rationale for discovery and preclinical profile of a selective SV2A ligand for epilepsy treatemt. Epilepsia. 2016, 57(4), 538-548.

[3] US Food and Drug Administration Brivaracetam [Prescribing information] **2016** [Accessed September 5,2020].https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&ApplNo=205836.

[4] Gillard, M.; Fuks, B.; Leclercq, K.; Matagne, A.; Binding characteristics of Brivaracetam, a selective, high affinity SV2A ligand in rat, mouse and human brain: relationshio to anti-convulsant properties. *Eur. J. Pharmaco.*, **2011**, 664(1-3), 36-44.

[5] Kasetelejin-Nolst Trenité, D.G.; Genton, P.; Parain, D.; Masnou, P.; Steinhoff, B.J.; Jacobs, T.; Pigeolet, E. Evaluation of Brivaracetam, a novel SV2A ligand, in the photosensitivity model. *Neurology*, **2007**, 69(10), 1027-1034.

[6] Biton, V.; Berkovic, S.F.; Abou Khalil, B.; Sperling, M.R.; Johnson, M.E.; Lu S. Brivaracetam as adjunctive treatment for uncontrolled partial epilepsy in adults; a phase lll randomized, double blind, placebo-controlled trial. *Epilepsia*. **2014**, 55(1), 57-66.

[7] Rolan, P.; Sargentini-Maier, M.L.; Pigeolet, E.; Stockis A. The pharmacokinetics, CNS pharmacodynamics and adverse event profile pf Brivaracetam after multiple increasing oral doses in healtjy men. Br. J. Clin. Pharmacol., 2008, 66(1), 71-75.

[8] Sargentini-Maier, M.L.; Rolan, P.; Connell, J.; Tytgat, D.; Jacobs, T.; Pigeolet, E.; Riethuisen, J.M.; Stockis, A. the pharmacokinetics, CNS pharmacodynamics and adverse event profile of Brivaracetam after single increasing oral doses in healthy males. *Br. J. Clin. Pharmacol*., 2007, 63(6), 680-688.

[9] Sargentini-Maier, M.L.; Espié, P.; Coquette, A.; Stockis, A. Pharmacokinetics and metabolism of 14C-Brivaracetam, a novel SV2A ligand, in healthy subjects. *Drug Metad. Sispos.*, 2008, 36(1), 36-45.

[10] Iqbal, M.; Ezzeldin, E.; Al-Rashhod, K.A. UPLC-MS/MS assay for identification and quantification of Brivaractema in plasma sample: Application to pharmacokinetic study in rats. *J. Chromatogr. B*, **2017**, 1060, 63-70.

[11] Atul Vasanth, D.; Rajkamal B. A Validated LC-MS/MS method for pharmacokinetic study of Brivaracetam in healthy rabbits. *Int. J. Pharm. Pharm. Sci.,* **2018**, 10(2), 24-29.

[12] Stockis, A.; Hartstra, J.; Mollet, M.; Hadi, S. Bipoavailability and bioequivalence comparison of Brivaractema 10,50,75, and 100 mg tablets and 100 mg intravenous bolus. *Epilepsia*, 2016, 57,1288-1293.

[13] Otoul, C.; Watanabe, S.; McCabe, S.; Stockis, A. Relative bioavailability and bioequivalence of Brivaracetam 10 mg/mL oral solution and 50-mg-fim-coated tablet. *Clin. Pharmacol. Drug Dev.*, **2017**, 6, 313-317.

[14] Susan M.; Roberto R.; Manuela C. Development and Validation of an UHPLC-MS/MS Assay for the Therapeutic Monitoring of Brivaracetam Plasma Concentrations in Patients with Epilepsy. Ther. Drug. Monit, 2020, 42(3), 445-451.

[15] Guidance for Industry; Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), (May 2018).

[16] Pilli, N.R.; Narayanasamy, S.; Xu, L.; Chockalingam, A.; Shea, K.I.; Stewart, S.; Rouse, R.; Patel, V.; Matta, M.K. A high-throughput bioanalytical assay to support pharmacokinetic interaction study of oxycodone and diazepam in Sprague Dawley rats. *RSC Advances*, **2020**, 10, 886-896.

[17] Narayanasmy, S.; Pilli, N.R.; Xu, L.; Chokalingam,A.; Shea, K.I.; Stewart, S.; Rouse, R.; Patel, V.; Matta, M.K. An alternating polarity switching assay for quantification of Oxycodone and topiramte: An application of LC-MS/MS method in support to PK/PD study in rodents. *J. Chromatog. B,* 2**019**, 1118, 93-100.

Table 1

LC-MS/MS Conditions:

LC Conditions		
Mobile Phase	0.05% Formic Acid solution (in Milli-Q/HPLC grade water) : Acetonitrile (15:85), (volume by volume)	
Column	Unisol, $C_{18,}$ 4. 6mm **X** 100mm, 5µm	
Autosampler Temperature	5°C	
Column Oven Temperature	30°C	
Mass Spectrometric Conditions		
	BRV	ISTD
Ionisation Mode	Positive	
Ion Transition, m/z	213.1/168.1	220.0/175.1
Ion-Source Temperature, °C	500	
Ion Spray Voltage, V	5500	
Nebulizer Gas, psi	50	
Turbolon Gas, psi	50	
Curtain Gas, psi	30	
Collision Gas, psi	5	
Cell Entrance Potential, V	10	10
Ion Declustering Potential, V	70	40
Ion Collision Energy, V	25	22
Collision Cell Eit Potential, V	12	12
Dwell Time, msec	200	200

Resolution	Unit	Unit
Acquisition Software	Analyst 1.7.0	

Table 2

Accuracy and Precision data for BRV:

Quality Control (Nominal conc.)	Concentration Calculated Mean ± SD (ng per mL)	Precision (%)	Accuracy (%)
Intra-Day (n=12)			
LLOQ (20 ng/mL)	20.35 ±0.91	4.49	101.75
LQC (60 ng/mL)	58.30 ±3.48	5.97	97.16
MQC (1800 ng/mL)	1851.21 ±58.22	3.14	102.85
HQC (3000 ng/mL)	3146.71 ±229.55	7.29	104.89
Inter-Day (n=18)			
LLOQ (20 ng/mL)	19.93 ±2.06	10.35	99.63
LQC (60 ng/mL)	58.25 ±4.98	8.55	97.08
MQC (1800 ng/mL)	1821.72 ±65.68	3.61	101.21
HQC (3000 ng/mL)	3084.55 ±205.92	6.68	102.82

Table 3

Stability data for BRV in plasma (n=6) :

Stability Experiment	QC Spiked Concentration (ng/mL)	Mean±SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Wet Extract stability (42 h)	60	56.30±3.14	5.58	95.61
	3000	2869.90±317.35	11.06	92.99
Auto-sampler (30 h)	60	5707±2.22	3.89	95.11
	3000	3113.89±210.51	6.76	103.80
Freeze and thaw (5 cycles)	60	60.05±2.60	2.60	100.09
	3000	3146.79±5.70	5.70	104.89
Bench Top (8 h)	60	59.70±5.34	8.94	99.50
	3000	3386.70±294.42	8.69	112.89
Long-term (70 days)	60	57.55±1.61	2.80	95.92
	3000	3146.79±163.72	5.20	104.89

Table 4

Pharmacokinetic Parameters of BRV after single oral administration of 100 mg BRV to healthy subjects (n=14, Mean ± SD) :

Parameter	Mean ± SD	
	Test	**Reference**
C_{max} (ng/mL)	1953.231±276.935	2009.464±333.824
t_{max} (h)	0.923±0.544	0.98±0.544
AUC_{0-t} (ng h/mL)	25596.192±3778.278	26082.843±4111.658
AUC_{0-inf} (ng h/mL)	26455.959±4003.353	27123.326±4364.324
$t_{1/2}$	9.754±1.216	10.126±1.496
Kel (h^{-1})	0.072±0.009	0.07±0.011

Brivaracetam Brivaracetam D_7

Figure 1. Chemical Structure of Brivaracetam and Brivaracetam-D_7 (ISTD).

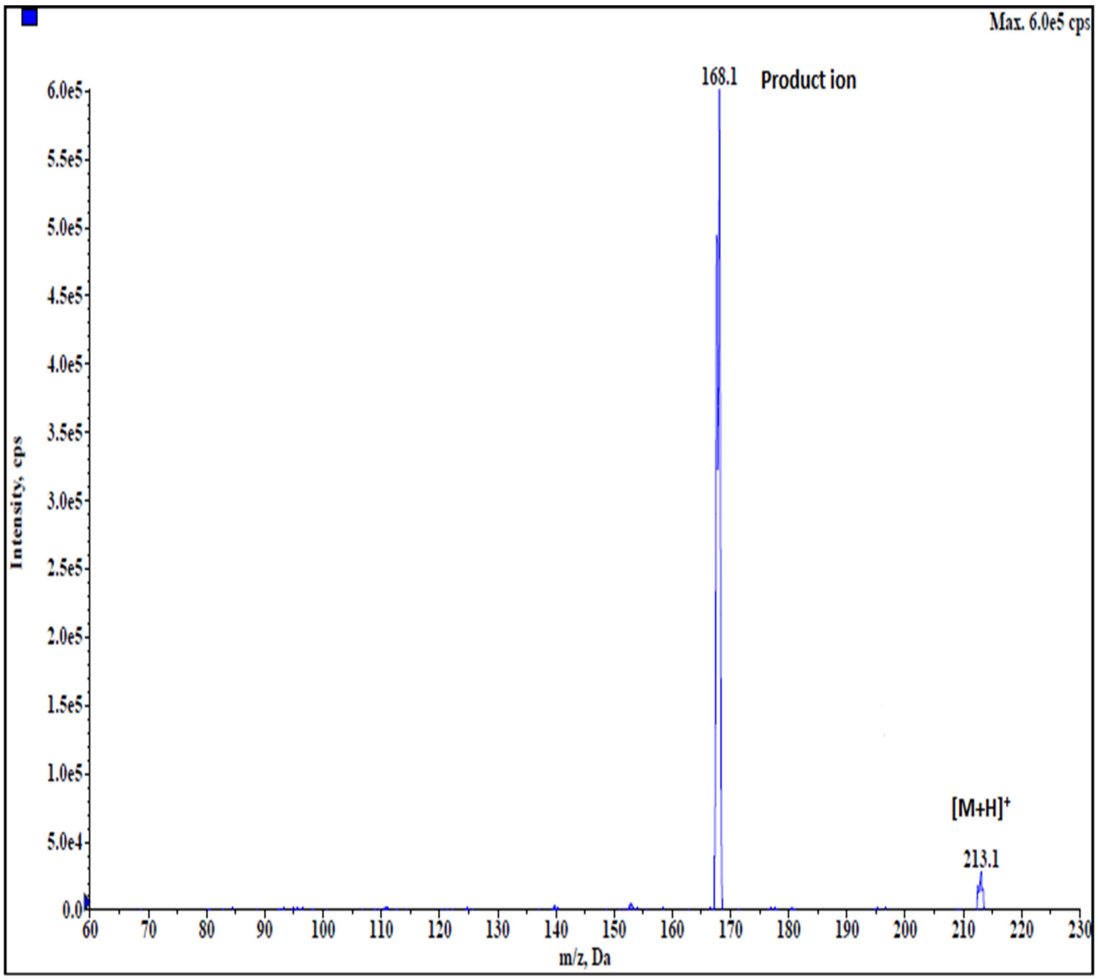

Figure 2A. Brivaracetam - Product-Ion Spectra.

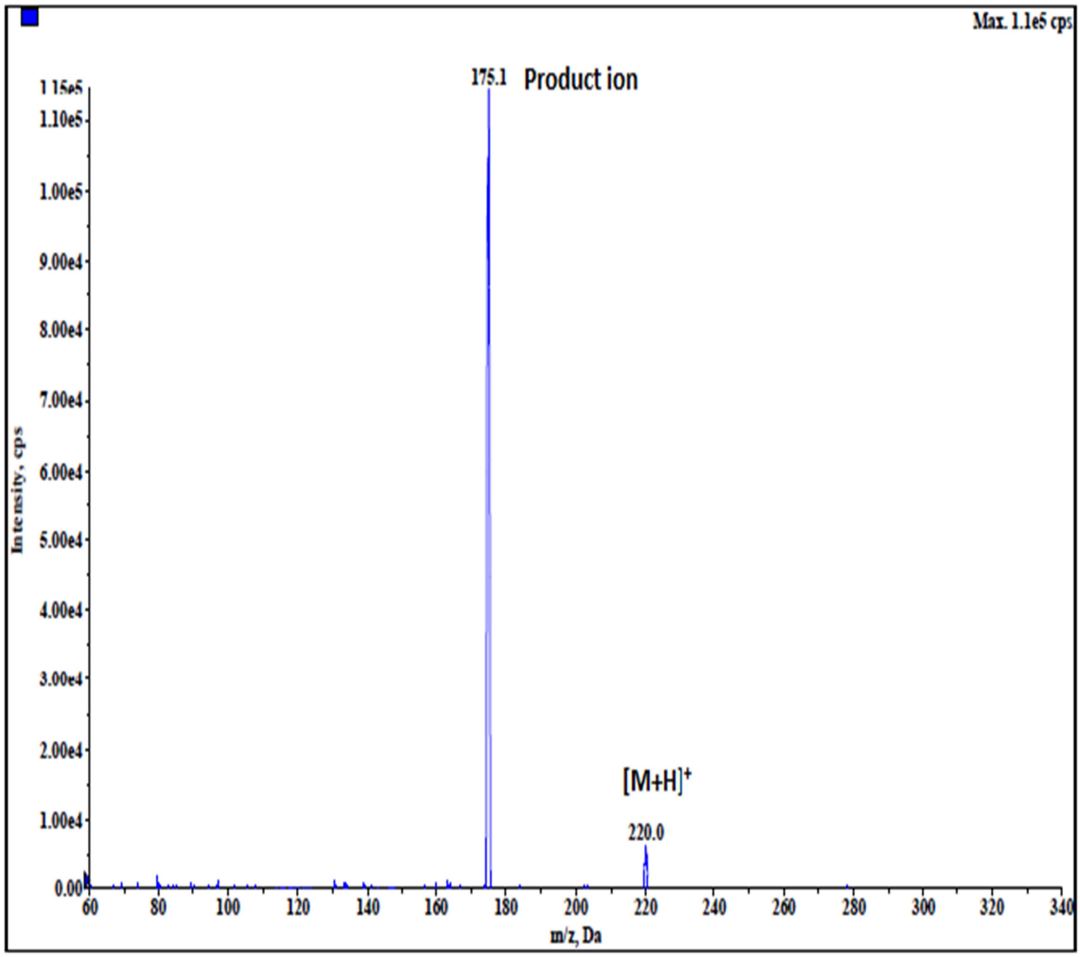

Figure 2B. Brivaracetam D_7 - Product-Ion Spectra.

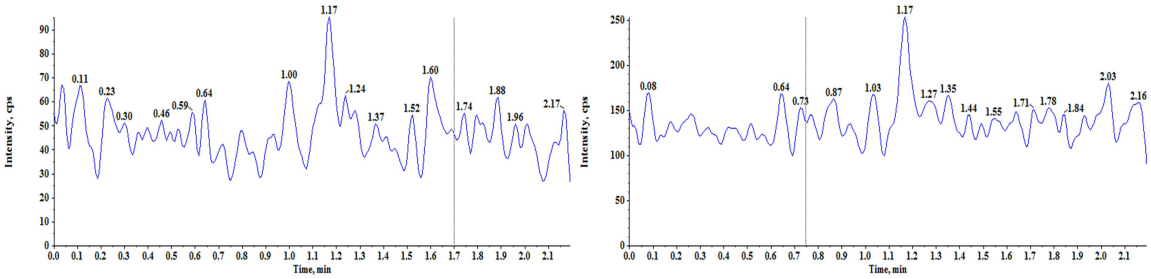

Figure 3. Blank Sample - Representative chromatograms (BRV - Left chromatogram and ISTD - Right chromatogram).

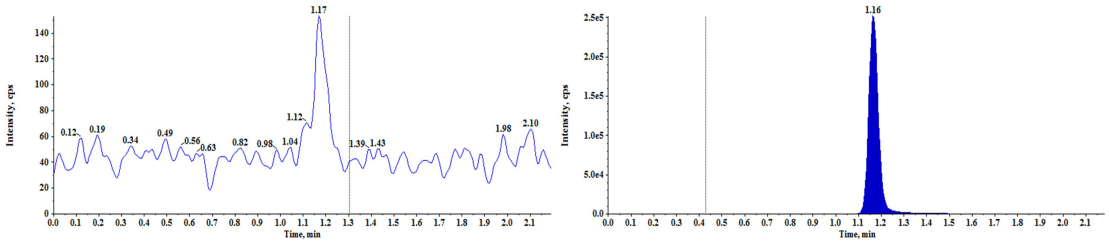

Figure 4. Blank with ISTD Sample - Representative chromatogram (BRV - Left chromatogram and ISTD - Right chromatogram).

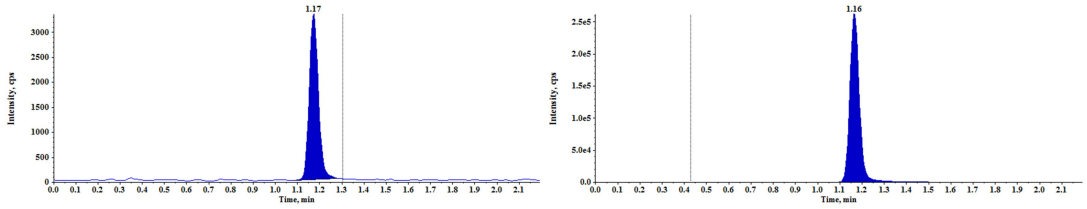

Figure 5. LLOQ sample - Representative chromatogram (BRV - Left chromatogram and ISTD - Right chromatogram).

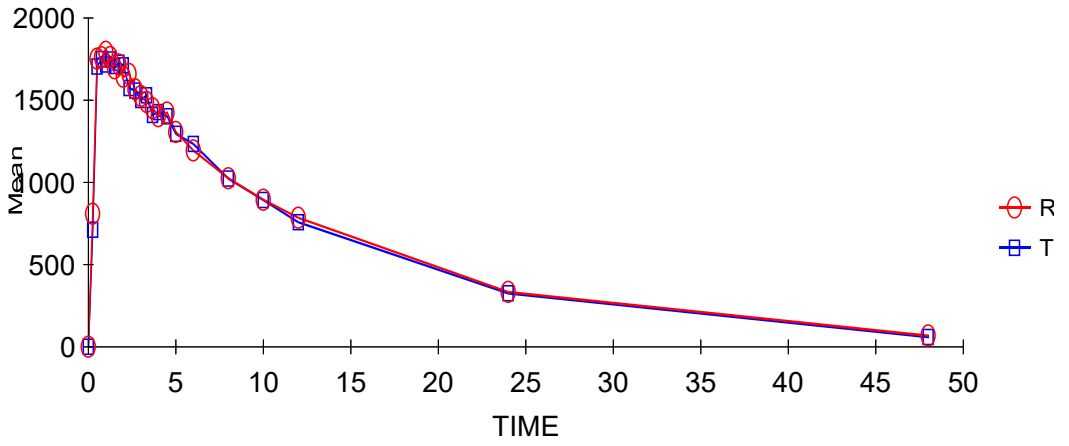

Figure 6. Mean plasma concentration versus time profile of BRV in human plasma following oral dose of BRV (100 mg tablet) in healthy volunteers (n=14).