International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 18, Issue 2, 2026

Original Article

SIMULTANEOUS METHOD DEVELOPMENT AND VALIDATION OF GABAPENTIN AND CARBAMAZEPINE IN RAT PLASMA USING LC-MS/MS AND ITS APPLICATION TO PHARMACOKINETIC STUDIES

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Received: 30 Aug 2025, Revised and Accepted: 30 Dec 2025

ABSTRACT

Objective: For the bioanalytical method of gabapentin and carbamazepine using tiagabine as the internal standard (IS), a simple, rapid, accurate, specific, and repeatable Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) approach was devised.

Methods: A Symmetry C18 column (150x4.6 mm, 3.5µ) and mobile phase of 0.1% formic acid in water and acetonitrile (70:30, v/v) are used in this article to summarise the latest advancements in bioanalytical LC-MS/MS procedures.

Results: Analysis was carried out within 7 min over a good linear concentration range from 6.0ng/ml to 240ng/ml (r^2= 0.9999±0.004) for Gabapentin and 2ng/ml to 80ng/ml (r^2=0.9998± 0.003) for Carbamazepine. Accuracy, precision, recovery, matrix effect and stability results were found to be within the suitable limits.

Conclusion: When used successfully for the examination of rat pharmacokinetic studies, the application indicates that all system appropriateness, specificity, linearity, and accuracy metrics are in excellent compliance with USFDA requirements.

Keywords: Gabapentin, Carbamazepine, LC-MS/MS, USFDA guidelines, Rat plasma

DOI: https://dx.doi.org/10.22159/ijap.2026v18i2.56696 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

The anticonvulsant [1, 2] medicine carbamazepine is used to treat epilepsy and neuropathic pain [3, 4]; it goes by many brand names, including Tegretol. Used in conjunction with other drugs, it is an adjuvant therapy for schizophrenia [5, 6] and a second-line agent in bipolar disorder [7, 8]. It seems that carbamazepine, along with phenytoin and valproate, is just as effective for both localised and widespread seizures [9, 10]. Myoclonic or absence seizures will not respond to it [11]. In an effort to lessen carbamazepine's negative systemic effects, researchers have created photoswitchable analogues of the medication to allow for local and on-demand light-based modulation of its pharmacological activity (photopharmacology). Analgesia with noninvasive [12] illumination *in vivo* was shown in a rat model of neuropathic pain by one of these light-regulated drugs, carbadiazocine, which is based on a bridging azobenzene or diazocine. Common conditions treated with carbamazepine include neuropathic pain and seizure disorders. When traditional antipsychotic therapy for schizophrenia has not been successful, it is sometimes used off-label as a second-line treatment for bipolar disorder or in conjunction with an antipsychotic [13, 14]. Nevertheless, there is evidence that it is not effective in treating schizophrenia. Myoclonic and absence seizures do not respond to it. Carbamazepine may be just as effective (as measured by people continuing to take the medication) and efficacious (as measured by the medicine reducing seizure recurrence and improving remission) as phenytoin and valproate, but each person's medication choice should be carefully considered because more research is needed to find out which medication is best for newly-onset seizures.

The anticonvulsant drug gabapentin is used to treat neuropathic pain and partial seizures of epilepsy; it is marketed under many trade names, including Neurontin. This medicine is often used to alleviate neuropathic pain, which may be caused by diabetic neuropathy [15, 16], postherpetic neuralgia [17, 18], or central pain,. About 30–40% of patients treated with gabapentin for diabetic neuropathy or postherpetic neuralgia report a significant improvement in their symptoms, indicating a moderate level of effectiveness. Similar to other gabapentinoid medications [19], gabapentin reduces the activity of the α2δ-1 protein, which is encoded by the CACNA2D1 gene. This protein was first recognised as an auxiliary subunit of voltage-gated calcium channels. See Pharmacodynamics, however, for more on it. Gabapentin decreases the release of excitatory neurotransmitters [20], mostly glutamate, by attaching to α2δ-1. Consequently, it lessens the overexcitation of neural networks in the brain and spinal cord. The two most prevalent adverse effects are drowsiness and vertigo [21, 22]. Some serious adverse effects include respiratory depression [23] and allergic responses [24, 25]. There is a warning about the increased risk of suicide with gabapentin and all other FDA-approved antiepileptic medications. People who have renal problems should take lower dosages.

Some literatures were available for gabapentin [26-28] and carbamazepine [29-31] individually. The lack of a simultaneous method for these two specific drugs in rat plasma, highlighting the clinical or research need for such a method. The study's overarching goal was to establish a novel, sensitive, and fast LC-MS/MS technique for the concurrent determination of gabapentin and carbamazepine in rat plasma, with tiagabine serving as an internal reference (In the simultaneous procedure two deuterated standards are used for analysis, It was critical to separate. So that I selected same category drug of Tiagabine as an internal standard).

MATERIALS AND METHODS

Chemicals and reagents

Merck (India) Ltd. of Worli, Mumbai, India, supplied the acetonitrile and formic acid, water (HPLC grade). We obtained all of the active pharmaceutical ingredients (APIs) for gabapentin, tigabine, and carbamazepine from Zydus Cadila Healtha Care Ltd in Ahmedabad to use as reference standards.

Equipment

An HPLC system, namely a Waters Alliance e2695 type, was used in conjunction with a QTRAP 5500 triple quadrupole mass

spectrometer. An operation was carried out using the ABSCIEX software [32].

LCMS

The examination was performed on a mass spectrometer QTRAP 5500 triple quadrupole instrument with a positive ion electrospray ionization interface. Working parameters of mass spectrometry after optimization as follows: Ion spray voltage 5500 V; temperature source 550 °C; Drying gas temperature 120-250 °C; Collision gas Nitrogen; Pressure 55 psi; Drying gas flow stream-5 ml/min; Declustering potential-40 V; Entrance potential-45 V; Exit potential-15 V; Capillary voltage-5500 V and Dwell time 1 sec respectively. MRM mode was employed to monitor ion pairs of mass: m/z 58.6342 → 172.2378, m/z 61.2047 → 237.2695 for Gabapentin and Carbamazepine, m/z 83.6591 → 376.5487 Tiagabine (Internal standard).

Chromatographic conditions

Symmetry C18 columns (150 x 4.6 mm, 3.5 micron) were used for isocratic mode at room temperature chromatographic separation. For the mobile phase, we used a 70:30 v/v combination of acetonitrile and 0.1% formic acid, with a flow rate of 1 ml/min. There was a 7 min runtime with an injection rate of 10µl.

Preparation of standard and internal control samples

Preparation of Gabapentin Parent Stock Solution (4800 ng/ml)

The standard solution of Gabapentin was prepared by accurately transferring 6 mg into 100 ml volumetric flask, dissolved the contents with diluent, and adjusted the final volume to the mark. The parent stock solution of the Gabapentin was prepared by transferring 0.8 ml to 10 ml volumetric flasks and adjusting the final volume with diluent.

Preparation of carbamazepine parent stock solution (1600 ng/ml)

The standard solution of Carbamazepine was prepared by accurately transferring 8 mg into 100 ml volumetric flask, dissolved the contents with diluent, and adjusted the final volume to the mark. The parent stock solution of the Carbamazepine was prepared by transferring 0.2 ml to 10 ml volumetric flasks and adjusting the final volume with diluent.

Preparation of standard stock solution (Gabapentin-480 ng/ml and carbamazepine-160 ng/ml)

Gabapentin and Carbamazepine combined working stock solution was prepared by transferring 1 ml of each drug working parent stock solution to a 10 ml volumetric flask, then diluting the mixture to the final volume with diluent.

Preparation of tiagabine (Internal standard) stock solution

The standard solution of Tiagabine was prepared by accurately transferring 6 mg into 100 ml volumetric flask, dissolved the contents with diluent, and adjusted the final volume to the mark. The parent stock solution of the Tiagabine was prepared by transferring 0.8 ml to 10 ml volumetric flasks and adjusting the final volume with diluent.

Preparation of standard solution

Here we used liquid-liquid extraction method for the extraction of drugs from plasma. We prepared the drug sample by transferring 0.5 ml of the combined standard stock solution into 2 ml centrifuged vials. To this 200 µl plasma, 500 µl internal standard stock, 300 µl acetonitrile, and 500 µl diluent were added, and the mixture was centrifuged at 4000 RPM for about 20 min. Later, the supernatant was collected and loaded into an HPLC vial.

Bioanalytical technique validation

Selectivity, sensitivity, linearity, accuracy, precision, matrix condition, recovery research, re-injection repeatability, and stability were all areas where the approach was shown to be valid [33, 34].

Selectivity

To ensure retention time selectivity and rule out interference, six separate rat plasma samples were analyzed.

Matrix effect

The matrix effect was determined by comparing the height-area ratio of six different drug-free plasma samples of gabapentin and carbamazepine. Six separate plasma lots were used in triplicate experiments conducted at MQC levels with an acceptable CV of ≤ 15%.

Precision and accuracy

Replicated analyses of internal control samples at four different quality control levels (LLOQC, LQC, MQC, and HQC) were used to get this result. The acceptable limit of CV for LQC, MQC, HQC is 15% and for LLOQ is 20%, the accuracy should be within 85%-115% for LQC, MQC, HQC and for LLQC is 80%-120%.

Recovery

The procedure involves extracting gabapentin and carbamazepine from six replicate samples at each internal control concentration. Standard height regions, both extracted and unextracted, are compared to one another to determine recovery [35].

Carry over

When a sample is diluted with blank matrix, any analyte that remains after the chromatographic system processes it with analyte concentrations over the Upper Limit of Quality Control (ULOQC) is considered a carryover [36].

Dilution integrity

Diluting a sample with blank matrix after spiked with an analyte concentration higher than the ULOQC should explain the dilution integrity [37].

Stability

Through contrasting the stability sample taken from a freshly prepared stock sample with the stability sample taken during the act of stock solution stability [38]. Plasma sample stability experiments were conducted with six replicates at both the LQC and HQC concentration levels. If the change is less than 15%, the analyte was deemed stable according to US FDA criteria [39]. We tested the integrity of spiking rat plasma that had been kept at room temperature for twenty-four hours. After being kept at room temperature in an auto sampler for twenty-four hours, the stability of spiked rat plasma was assessed. By comparing the extract plasma samples that were injected immediately with those that were re-injected after storage with wet extract stability at room temperature after 12 h and 18 h at 2-8 °C, the auto sampler stability (LQC, MQC, and HQC) was assessed. To test the repeatability of reinjection, we compared plasma samples that were extracted and injected right away with those that were reinjected after being stored in the dry extract stable at room temperature for 12 h and 18 h at-20±3 °C. The stability of the samples was tested by comparing them to newly spiked internal control samples and steadiness samples that had been frozen at-31 °C and thawed three times. The stability test was run for 7 d at 7 °C. The concentrations obtained after 24 h were compared to the starting concentration in order to evaluate the stability over the long term.

Pharmacokinetic study

Biological E Limited in Hyderabad, India, supplied six white New Zealand rats (about 250 g) for *in vivo* pharmacokinetic investigations. The Animal Ethics Committee of the Institute gave their stamp of approval to the study's protocol (Reg. No: 1250/PO/RcBi/S/09/CPCSEA). The animals were housed in similar laboratory conditions with access to endive, carrots, fresh corn (few amounts only). The animals were kept at a temperature of 21-24 °C and humidity was 50-55%. Before experimentation, all animals were made to fast overnight and had water ad libitum. Each rat was given the samples when it was fasting. Blood was collected into centrifugal

radioimmunoassay vials containing EDTA from each animal at specified intervals through *retro*-orbital plexus, centrifuged at 4000 rpm for about 15–20 min to collect the supernatant plasma. Blood samples were collected from rats at 1, 2, 3, 4, 8, 12, 16, and 20 h after oral administration of Gabapentin and Carbamazepine. Following a temperature range of 2-8 °C, the blood was spun at 4000 rpm for 20 min. The transparent plasma supernatant was collected and kept at-30 °C until it could be analyzed. We used a newly developed analytical approach to determine the drug concentration in the plasma samples after subjecting them to liquid-liquid phase extraction. The animals were taken back to the animal shelter for rehabilitation after the research.

Plasma concentration data were used to assess the pharmacokinetic characteristics for the oral administration of carbamazepine and gabapentin. Standard pharmacokinetic parameters include area under the curve (AUC), maximum concentration (Cmax), time to peak concentration (Tmax), and the time at which Cmax occurred. From zero to the infinity point of the concentration-time curve, the data was measured using the trapezoidal rule approach. From the graph, we were able to get Cmax and Tmax. The mean±SD is used to represent all values. (SD) stands for standard deviation.

RESULTS AND DISCUSSION

Using electrospray ionisation yields the best results when using this method's air pressure chemical ionisation mode. Gabapentin and carbamazepine, when administered in the positive ion mode with a mobile phase flow rate of 10 µl/min, provide excellent sensitivity and signal stability when used continuously with electro spray ions.

Specificity and selectivity

Using optimized parameters, the selectivity and specificity of the method were determined by injecting six different animal-extracted blank plasma samples and plasma spiked with internal standards into the LC-MS/MS. The results (fig. 1-3) showed that plasma components don't affect the analyte's retention time value ranges or internal standards. This means that the new method is selective.

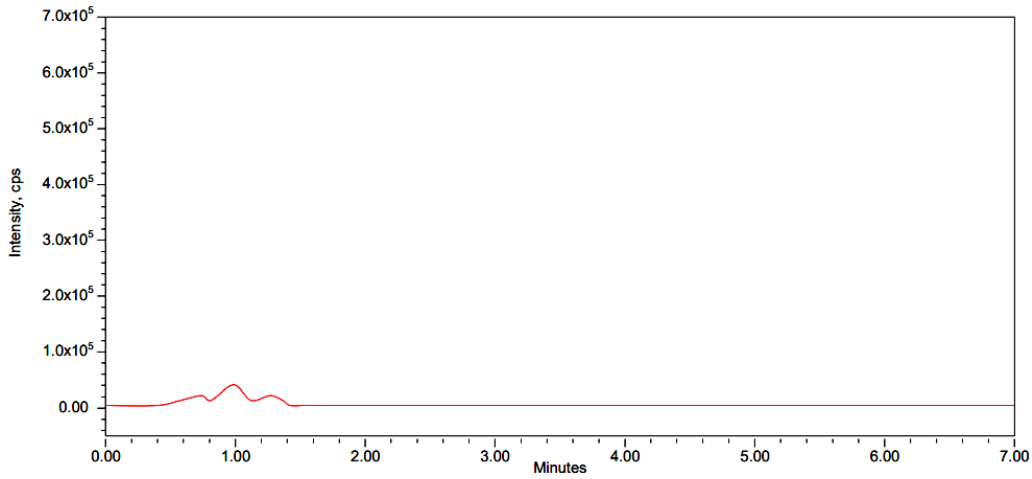


Fig. 1: **Blank chromatogram**

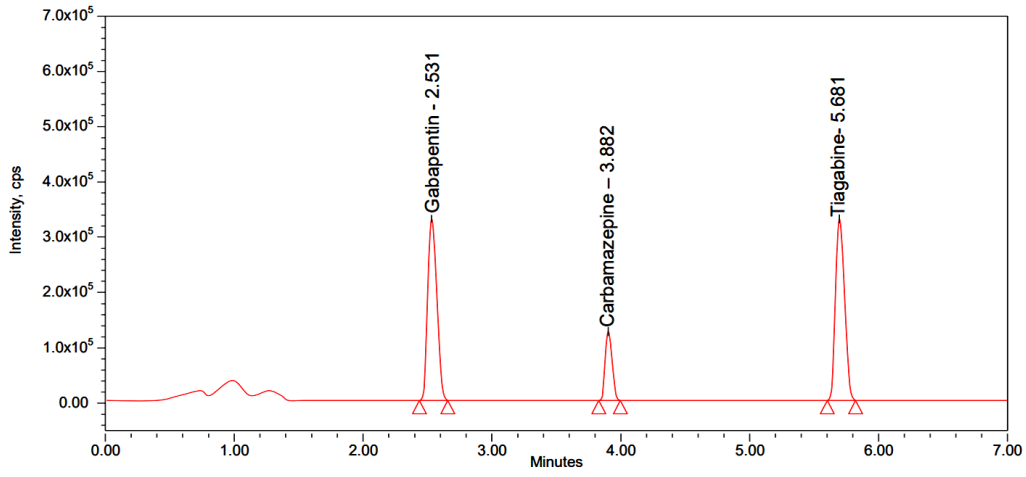


Fig. 2: Standard chromatogram

Matrix effect

Under these conditions, the matrix impact [40] on analyte ionisation is within an acceptable range, as the percent RSD (Relative Standard Deviation) for within-signal ion suppression/enhancement for gabapentin and carbamazepine in LC-MS/MS was one percent. Gabapentin had a LQC of 96.58 and an HQC of 98.11 in the matrix effect, whereas carbamazepine had 96.63 and 97.94%. The relative CVs for the two medications at the LQC and HQC levels were 0.89 and 0.10 and 1.74 and 0.27, respectively. This result shows that the analyte's ionization is affected by the matrix effect within the acceptable range.

Linearity

Concentration had a direct correlation with the peak area ratio of the standards used for calibration. Both gabapentin and carbamazepine have concentration ranges of 6–240 ng/ml and 2-80 ng/ml, respectively. Table 1 shows the results of the linearity test for

gabapentin and carbamazepine, and fig. 4 shows the calibration plots for both drugs [41]. The correlation coefficient for gabapentin and carbamazepine was determined to be 0.999, and the calibration curves seemed to be linear.

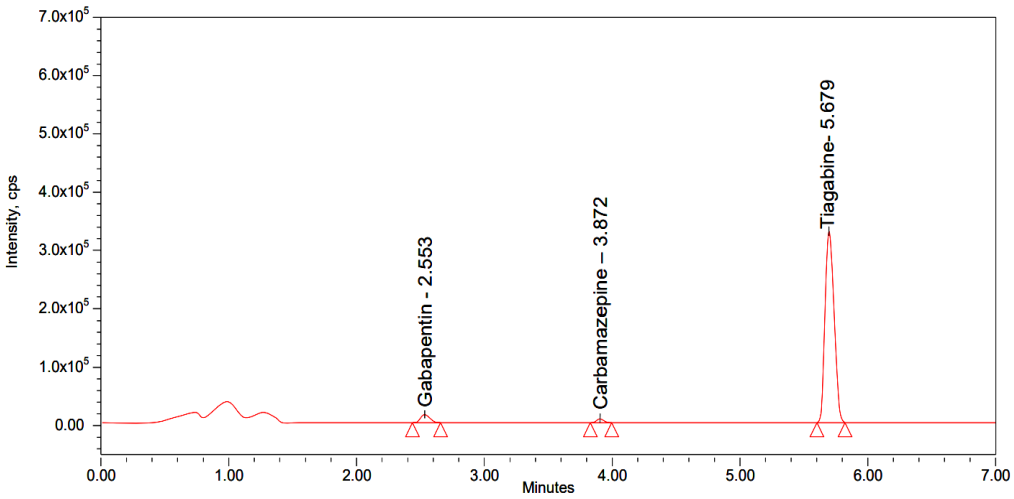
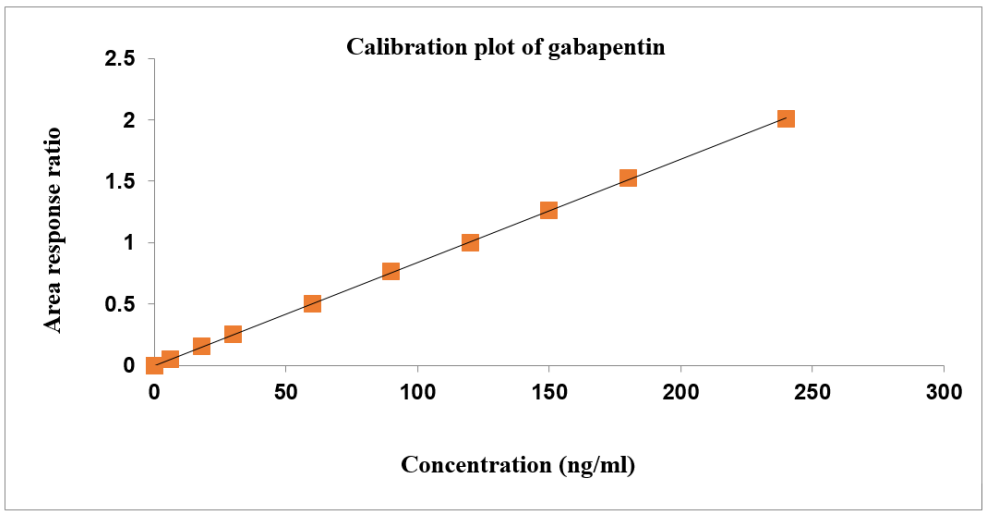


Fig. 3: LLOQ chromatogram



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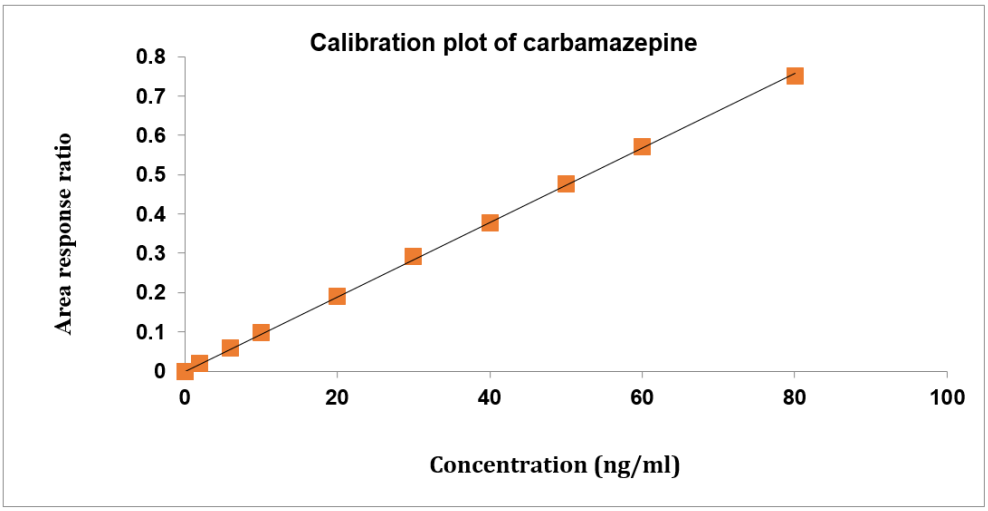

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Fig. 4: Calibration plots of (A) Gabapentin and (B) Carbamazepine

Table 1: Results of linearity

Linearity	Gabapentin				Carbamazepine			
	Conc. (ng/ml)	Analyte Area	IS Area	Area response ratio (Analyte/IS)	Conc. (ng/ml)	Analyte area	IS Area	Area response ratio (Analyte/IS)
1	6	0.174	3.254	0.053	2	0.065	3.254	0.020
2	18	0.507	3.235	0.157	6	0.193	3.235	0.060
3	30	0.823	3.247	0.253	10	0.318	3.247	0.098
4	60	1.639	3.265	0.502	20	0.621	3.265	0.190
5	90	2.466	3.229	0.764	30	0.942	3.229	0.292
6	120	3.268	3.258	1.003	40	1.229	3.258	0.377
7	150	4.095	3.242	1.263	50	1.543	3.242	0.476
8	180	4.921	3.231	1.523	60	1.848	3.231	0.572
9	240	6.576	3.274	2.009	80	2.461	3.274	0.752
Slope				0.0084	Slope			0.0095
Intercept				0.00330	Intercept			0.00109
CC				0.99993	CC			0.99982

Precision and accuracy

The accuracy and precision [42] were determined by combining the individual test results from several internal control samples. The statistics presented made it very clear that the approach was spot on and productive. Tables 2 and 3 provide the accuracy findings for gabapentin and carbamazepine, respectively. Results for gabapentin range from 94.54 to 98.53 on the quality control samples, whereas those for carbamazepine are 94.46 to 98.86. The percentage of gabapentin and carbamazepine in the total internal control samples is less than 5%. The acceptable limit of recovery was 85-115% for LQC, MQC, HQC and for LLQC the limit is 80-120%. [We used Microsoft Office Excel's function of STDEV to calculate standard deviation and we get following results and %Accuracy also the same value as we use the formula (% measured/nominal *100)].

Recovery

The recoveries for Gabapentin and Carbamazepine at LQC, MQC and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for Gabapentin (95.76%-98.10%) and Carbamazepine (95.55%-98.64%) at LQC, MQC and HQC levels and % CV ranged from 0.05-0.83 for Gabapentin and 0.70-1.34 for Carbamazepine. The results demonstrated that the bioanalytical method had good extraction efficiency [44].

Table 2: Precision and accuracy of gabapentin

S. No.	HQC	MQC	LQC	LLQC
	Nominal concentration (ng/ml)			
	180	120	18	6
	Measured concentration (ng/ml)			
1	177.8	117.7	17.2	5.6
2	175.7	118.4	17.6	5.7
3	178.8	118.1	17.2	5.8
4	178.1	118.9	17.6	5.7
5	176.4	117.3	17.4	5.6
6	177.2	117.2	17.3	5.5
n	6	6	6	6
mean	177.3	117.9	17.4	5.7
SD	1.12463	0.65253	0.19994	0.10087
% CV	0.63	0.55	1.15	1.78
% Accuracy	98.50%	98.25%	96.67%	95.00%

Table 3: Precision and accuracy of carbamazepine

S. No.	HQC	MQC	LQC	LLQC
	Nominal concentration (ng/ml)			
	60	40	6	2
	Measured concentration (ng/ml)			
1	59.2	39.4	5.8	1.8
2	59.0	39.7	5.7	1.9
3	59.2	39.5	5.8	1.9
4	59.1	39.6	5.7	1.9
5	59.1	39.4	5.7	1.9
6	59.1	39.5	5.6	1.9
n	6	6	6	6
Mean	59.1	39.5	5.7	1.9
SD	0.07037	0.10655	0.07037	0.03364
% CV	0.12	0.27	1.23	1.79
% Accuracy	98.50%	98.75%	95.00%	95.01%

Ruggedness

In all four quality control (HQC, LQC, MQC, and LLQC) samples, the percentage recoveries and percent CV of gabapentin and carbamazepine showed up within acceptable ranges when measured with two separate analysers using two separate columns. The method's findings demonstrated its robustness. For gabapentin, the percent recoveries varied between 96.79%-98.13%, while for

carbamazepine, they ranged from 95.01% to 97.96%. For gabapentin, the %CV values were 0.42-0.49, while for carbamazepine, they were 1.11-1.27. The method's findings demonstrated its robustness.

Auto sampler carryover

Injecting blank rat plasma samples with LLQC and ULQC sequentially at the retention durations of gabapentin and carbamazepine did not result in an observable peak area response. Auto sampler carryover is not shown by this approach.

Stability

A solution stability study was conducted on gabapentin and carbamazepine, which were produced using diluent and then stored in a refrigerator at 2-8 °C. Stock solutions that were produced 24 h before to use were paired with fresh stock solutions. Both the bench top and auto sampler plasma stability remained constant during twenty-four hours, with the auto sampler maintaining stability for twenty-four hours at 20 °C. Future stability testing confirmed that both gabapentin and carbamazepine were stable for up to 24 h when stored at-30 °C. In long-term stability, the samples were stable up to 21 d, but on going to 28 d Gabapentin was not stable in LQC condition and Carbamazepine also not stable in both LQC and MQC conditions. In tables 4, 5, we can see the overall stability findings of carbamazepine and gabapentin.

***In vivo* pharmacokinetic evaluation**

Fig. 5 shows the temporal patterns of plasma concentrations of gabapentin and carbamazepine in rats. The experimental formulations in both instances showed a bell-shaped curve on the graph. The fact that gabapentin and carbamazepine were detectable in the blood for 8 and 16 h after oral and intravenous dosing, respectively, suggests that the formulation was successful in releasing the drugs [44].

The results of the calculations of the pharmacokinetic parameters Cmax, Tmax, T1/2, AUC0-t, and AUC0-∞ are shown in table 6. Gabapentin had a Cmax of 116.360 ng/ml and carbamazepine a Cmax of 39.463 ng/ml. It was determined that the Tmax for carbamazepine was 8 h and that for gabapentin it was 3 h. Gabapentin and carbamazepine had t½ values of 8 h and 16 h, respectively. Gabapentin had an AUC0-t of 439 ng-h/ml and carbamazepine of 315 ng-h/ml. Table 6 displayed the pharmacokinetic parameters.

Table 4: Stability results of gabapentin

Stability experiment spiked plasma		Mean area±SD	% CV	%Recovery
Bench top stability	LQC	17.3±0.18708	1.08	96.11
	MQC	117.9±0.42622	0.36	98.25
	HQC	177.4±0.51153	0.29	98.56
Auto sampler stability	LQC	17.4±0.25626	1.46	96.67
	MQC	118.1±0.30332	0.26	98.42
	HQC	177.6±0.26077	0.15	98.67
Long term (Day28) stability	LQC	15.0±0.26394	1.76	83.33
	MQC	102.1±0.25820	0.17	85.08
	HQC	153.6±0.34641	0.23	85.33
Wet extract 18 H stability	LQC	17.2±0.22527	1.99	95.56
	MQC	115.8±0.35777	0.31	96.50
	HQC	173.2±0.28810	0.17	96.22
Dry extract 18 H stability	LQC	17.2±0.27869	1.60	95.56
	MQC	115.7±0.18708	0.16	96.42
	HQC	172.9±0.31411	0.18	96.06
Freeze thaw stability	LQC	17.4±0.27325	1.56	96.67
	MQC	118.1±0.19408	0.16	98.42
	HQC	177.6±0.20976	0.12	98.67
Short term stability	LQC	16.9±0.28284	1.66	93.89
	MQC	114.4±0.21602	0.19	95.33
	HQC	171.3±0.20412	0.12	95.17

Mean±SD (n=6)

Table 5: Stability results of carbamazepine

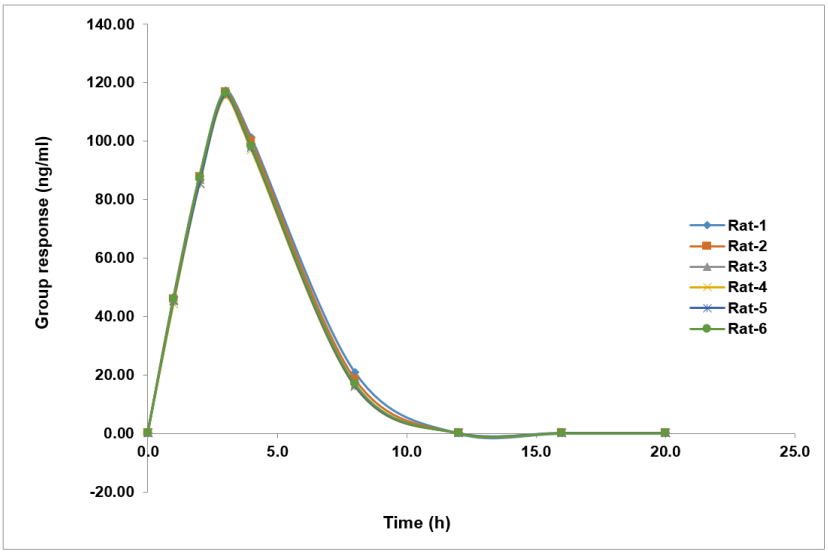
Stability experiment spiked plasma		Mean area±SD	% CV	%Recovery
Bench top stability	LQC	5.8±0.10328	1.77	96.67
	MQC	39.4±0.18619	0.47	98.50
	HQC	59.3±0.18708	0.32	98.83
Auto sampler stability	LQC	5.7±0.08615	1.43	95.00
	MQC	39.5±0.27869	0.71	98.75
	HQC	59.1±0.17889	0.30	98.50
Long term	LQC	5.0±0.08944	1.79	83.33
(Day 28) stability	MQC	33.7±0.26077	0.78	84.25
	HQC	52.1±0.28810	0.25	86.83
Wet extract 18 h stability	LQC	5.7±0.08944	1.54	95.00
	MQC	38.5±0.22804	0.59	96.25
	HQC	57.8±0.20012	0.35	96.33
Dry extract 18 h stability	LQC	5.6±0.07528	1.34	93.33
	MQC	38.2±0.21370	0.56	95.50
	HQC	57.9±0.22804	0.40	96.50
Freeze thaw stability	LQC	5.8±0.07528	1.30	96.67
	MQC	39.0±0.14142	0.36	97.50
	HQC	59.1±0.13784	0.23	98.52
Short term stability	LQC	5.6±0.08165	1.47	93.38
	MQC	37.9±0.11690	0.31	94.75
	HQC	57.1±0.14720	0.26	95.17

n=6

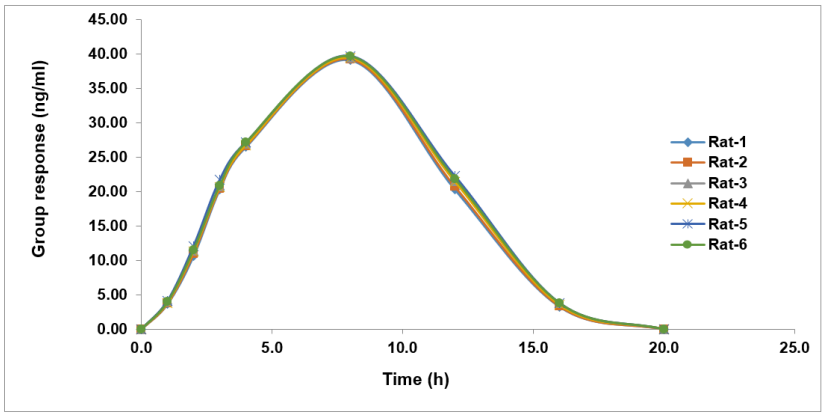
Table 6: Pharmacokinetic parameters of gabapentin and carbamazepine

Pharmacokinetic parameters	Gabapentin	Carbamazepine
AUC_{0-t}	439 ng-h/ml	315 ng-h/ml
C_{max}	116.360 ng/ml	39.463 ng/ml
$AUC_{0-\infty}$	566 ng-h/ml	361 ng-h/ml
t_{max}	3 h	8 h
$T_{1/2}$	4.98 h	3.5 h

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, AUC_{0-t}: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration



A



B

Fig. 5: Mean plasma concentration-time profile of (A) Gabapentin and (B) Carbamazepine

CONCLUSION

To determine carbamazepine and gabapentin in rat plasma, the first ever validated technique was a highly sensitive HPLC-ESI-LCMS/MS. In this case, we have a bioanalytical approach that is robust, rapid, and repeatable. Following USFDA requirements, this approach was verified. In order to see the studied analyte in bodily fluids and conduct pharmacokinetic research, a simple and effective technique was devised.

ACKNOWLEDGEMENT

I thankful to my guide for encouragement and supporting to finish this research work.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All of the drug-related material has been compiled by Mubeena. The research samples and manuscript preparation were done by Bharath, Sree Ramudu, and Srividya. The data was checked by Ramachandran, who also read the manuscript.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest

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