Section A-Research paper

BIOANALYTICAL METHOD DEVELOPMENT AND THERAPEUTIC DRUG MONITORING OF CARBAMAZEPINE

Prathyusha Thunla[1], Akiladevi.D[2]

[1] Department of Pharmacy Practice Vaagdevi Institute of Pharmaceutical Sciences, Warangal-506005, India.

[2] Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology, and advanced studies. Pallavaram, Chennai-600117. TN, India.

Email Id:ushapharmd10@gmail.com

Abstract

Background: Carbamazepine is a standard antiepileptic medication that has structural similarities with tricyclic antidepressants. Carbamazepine in plasma may be detected using a variety of techniques, including Therapeutic Drug monitoring (TDM). Carbamazepine TDM has been touted as beneficial in a number of trials, although definitive criteria for TDM have yet to be established.

Method: To evaluate Carbamazepine concentrations in human plasma, a bioanalytical technique was devised and validated using liquid chromatography (LC). Mean blood levels of Carbamazepine were 4995.54ng/mL in men and 3485.92ng/mL in females, according to an analysis of plasma concentrations in 84 epileptic participants receiving 100 to 400 mg/dose.

Results: Inter- and intra-day precision at 70 ng/ml, 550 ng/ml, and 950 ng/ml for Carbamazepine are 0.02-0.04, 0.03-0.08, respectively, whereas for Diclofenac these values are 0.01-0.03 and 0.02. All of these values were calculated in accordance with ICH criteria. The findings of TDM for Carbamazepine with Diclofenac as a co-medication were statistically significant, with the mean blood levels reaching steady state in 84 patients using the medicine for a prolonged period of time. Mean serum concentrations were 4995.54ng/ml in women and 3485.92ng/ml in males. The total dosage range of the study was around 100 - 400 milligrams.

Conclusion: The proposed technique for determining Carbamazepine concentration was quick, precise, and accurate. Consequently, randomized, controlled trials (RCTs) in a wider population with a broad range of dose to correspond with the population's overall serum levels and several co-medications for dose modifications to achieve the maximal safe dosage in the individual are anticipated.

Keywords: Bioanalytical method development, validation, Epilepsy, Carbamazepine, Diclofenac, Therapeutic drug monitoring.

1. Introduction

Epilepsy has an incidence of 0.5% to 1% in industrialized nations, making it the most frequent neurological disorder worldwide. As a consequence of a mismatch in the CNS's excitatory and inhibitory circuits, seizures may occur. There is still much mystery about epilepsy's root cause (some of the factors that might cause this include birth trauma, CNS infections, head trauma, and malignant brain tumors). Treating epilepsy successfully requires both an accurate diagnosis of the condition and the implementation of a suitable treatment plan. About 70% of individuals may achieve complete seizure control through monotherapy. Selecting an antiepileptic medication requires consideration of the characteristics of each patient's case. A number of factors, including the individual's age, the presence of other conditions, and the potential for medication interactions, must be considered while treating epilepsy (Grzek et al., 2021).

1.1 Carbamazepine

Carbamazepine is used for a wide variety of conditions, including epilepsy, trigeminal neuralgia, and acute mania and mania-like episodes related to bipolar I disorder. Carbamazepine (CBZ), also known as 5-H-dibenze[b,f]azepine-5-carboxamide (Figure 1), is a tricyclic lipophilic molecule and a first-line antiepileptic medication for the management of both simple and complicated partial seizures. Nearly majority of the drug is broken down in the liver, with just tiny amounts passing through the kidneys unaltered. Therapeutic amounts of 6-12 µg/ml have been recorded, while large fluctuations are possible. Carbamazepine-10,11-epoxide is an active metabolite of CBZ that correlates considerably to its effectiveness and toxicity and displays therapeutic properties as an antiepileptic drug, albeit at lower doses than CBZ. This limits the usage of a known therapeutic range for CBZ concentration. Literature reports a number of CBZ-detection strategies, most notably chromatographic approaches (Budakova et al., 2008; Oh et al., 2006; Franceschi and Furlanut, 2005; Raggi et al., 2000; Elizabeth et al., 2007; Hemenway et al., 2010; Yoshida et al., 2006).

Figure 1: Structure of Carbamazepine

1.1.1 Mechanism

By affecting voltage-gated sodium channels (VGSC), carbamazepine suppresses electrical impulses and slows down synaptic activity. There is speculation that carbamazepine, like certain other anti-convulsants, interacts with the alpha subunit of

VGSC, precisely in a pocket established by the pore's outermost loops and the membrane of domain IV. Carbamazepine has been reported to reduce action potential production because it prevents sodium channels from reversibly activating, resulting to fewer channels being open (Maan et al., 2021).

1.1.2 Dose

Tablets of 100mg and 200mg dosage, as well as extended-release tablets of 100mg, 200mg, 300mg, and 400mg, are among the several forms of Carbamazepine on the market. Starting doses for adults are 200 mg twice day and for children under 12 it is 100 mg twice daily, with dosages adjusted upwards to the minimal effective dose over time. Treatment of epilepsy with 800 to 1200 milligrams per day is minimally beneficial in adults and children over the age of 12. Treatment of epilepsy with 400–800 milligrams per day is beneficial for children aged 6–12 years old.

1.2 Diclofenac

Diclofenac sodium, also called N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (Figure 2), is an NSAID that relieves pain, fever, and inflammation. Though it has a low solubility in water, the salt form of the molecule is easily absorbed by the body via the digestive system. It has a more focused effect on COX-2 but also has an off-target effect on COX-1. Patients with epilepsy who are resistant to currently available AEDs may benefit from combining NSAIDS with these medications, since their usage has been shown to reduce the frequency and severity of seizures (Javed et al., 2021).

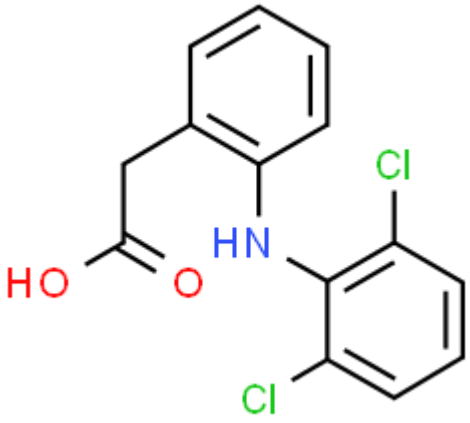

Figure 2: Structure of Diclofenac

Since there is a lack of information on the efficacy of Diclofenac in combination with the gold standard antiepileptic drug (AED), Carbamazepine, for the treatment of epilepsy, the current research aims to develop and verify a bioanalytical technique for Carbamazepine and Diclofenac, with pantoprazole serving as an internal standard (IS).

In order to ascertain the blood levels of Carbamazepine, therapeutic monitoring was performed in patients with epilepsy.

2. Materials & Methods

2.1 Sampling from Subjects

All patients gave their informed permission before any treatments were performed, as required by our hospital's ethics guidelines and the Helsinki Declaration (as updated in 2000). Over the course of a year, plasma samples were taken for regular monitoring of antiepileptic medicines. Eighty-four individuals' serum samples were analyzed for Carbamazepine levels.

2.2 Inclusion criteria

1. Long-term therapy with chosen antiepileptic drugs (Carbamazepine and Diclofenac) and comedication for a minimum of 1-month,

2. Between the age of 20-60 yrs,

3. No dose adjustments to the anti-epileptic therapy in the last three weeks

2.3 Exclusion criteria

1. Women who are expecting or nursing a child.

2. Individuals that are younger than twenty years of age

3. Those who are not interested in taking part in the research as patients.

4. Senior citizens (those aged 60 and above).

5. Patients with concomitant conditions such as hypertension and DM are not eligible.

6. No evidence of liver or kidney dysfunction.

2.4 Chemicals and Reagents

Drugs like Carbamazepine and Diclofenac were obtained from Dr. Reddy's, while other chemicals like potassium orthophosphate, acetonitrile (ACN), and orthophosphoric acid (OPA) were obtained from Merck.

An adequate quantity of Carbamazepine, Diclofenac, and pantoprazole (IS) was dissolved in acetonitrile (ACN) to produce stock solutions with a 1 mg/mL drug concentration. The maximum storage time for these solutions was 4 weeks at 4 degrees Celsius. For use in experiments, 1000 ng/mL working solutions were formed through dilution of stock solutions with ACN.

2.5 Instrumentation and Chromatographic Conditions

The chromatographic setup consists of a Rheodyne Injector with a 20 µL fixed volume, an LC 20AD, and an SPD 20A detector (Shimadzu). For managing the system and

gathering and analyzing information, LC Solutions (Ver 1.25 software was utilized (Shimadzu). Carbamazepine, Diclofenac, and Pantoprazole were chromatographically separated on a Phenomenex C18 column. The pH of the mobile phase, which comprised of 10mm Potassium orthophosphate buffer and ACN (55:45) was fixed to 4.00±0.05 with the help of orthophosphoric acid (OPA) for the isocratic elution. Runtime is 10.00 minutes at a flow rate of 1 ml/min with a working wavelength of 220 nm.

2.6 Sample Extraction

In a 2 mL Eppendorf tube, we mixed 50 µL of serum with 5 µL of ACN and 15 µL (1000 ng/mL) of IS and centrifuged it for ten minutes at 10000 rpm. After letting the contents settle to the bottom, 20 µL of the supernant was injected.

2.7 Validation of the Method

Ten sets of standards with varying amounts of Carbamazepine and Diclofenac in drug-free serum were used to calculate the intra-day precision. Using 10 separate test runs, three diverse concentrations were utilized to study the technique's inter-day precision trend. Accuracy for the two drugs was determined at 650ng/ml by calculating the mean of peak areas obtained. The formula employing the standard deviation (σ) of the response and the slope were found out to calculate the limits of detection (LOD) and quantification (LOQ). We used following formulas to determine the LOD and LOQ,

$$LOD = \frac{3.3 \times \sigma}{Slope}$$

$$LOQ = \frac{10 \times \sigma}{Slope}$$

2.8 Statistical Analysis

Concentrations of Carbamazepine were measured in patients and their means and standard deviations were determined. Two groups (female and male) were compared using Welsch's t-test and one-way ANOVA.

3. Results

3.1 Chromatographic conditions

The chromatograms obtained for in vivo, in vitro and blank samples were recorded, as shown below (Figure 3- Figure 6).

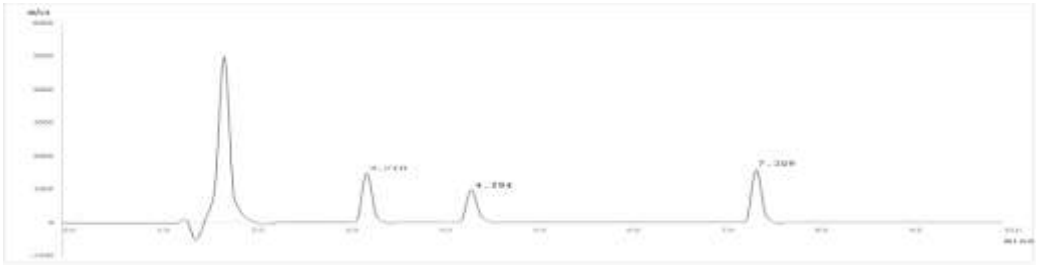

Figure 3: Chromatogram for Invivo sample (I)

SHIMADZU LC REPORT

SAMPLE: INVIVO

FLOW RATE: 10ml/min

INJECTION VOLUME: 20µL

WAVLENGTH: 220nm

RUNTIME:10.0MINS

RETENTION TIME

CARBAMZEPINE: 3.210

DICLOFENAC: 7.309

PANTOPRAZOLE (IS): 4.394

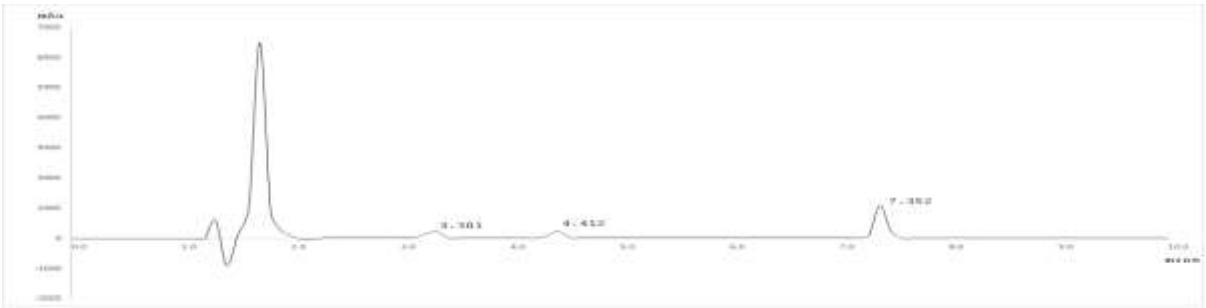

Figure 4: Chromatogram for Invivo sample (II)

SHIMADZU LC REPORT

SAMPLE: INVIVO

FLOW RATE: 10ml/min

INJECTION VOLUME: 20µL

WAVLENGTH: 220nm

RUNTIME:10.0MINS

RETENTION TIME

CARBAMZEPINE: 3.301

DICLOFENAC: 7.352

PANTOPRAZOLE (IS): 4.412

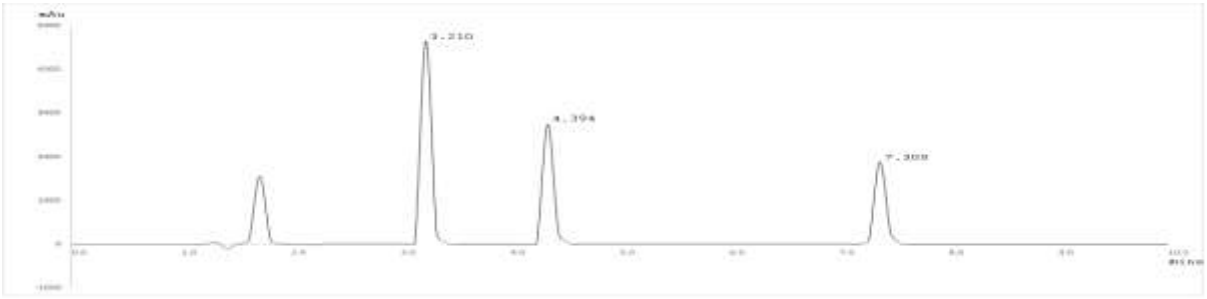

Figure 5: Chromatogram for invitro sample

SHIMADZU LC REPORT

SAMPLE: INVITRO

FLOW RATE: 10ml/min

INJECTION VOLUME: 20µL

WAVLENGTH: 220nm

RUNTIME:10.0MINS

RETENTION TIME

CARBAMZEPINE: 3.210

DICLOFENAC: 7.309

PANTOPRAZOLE (IS): 4.394

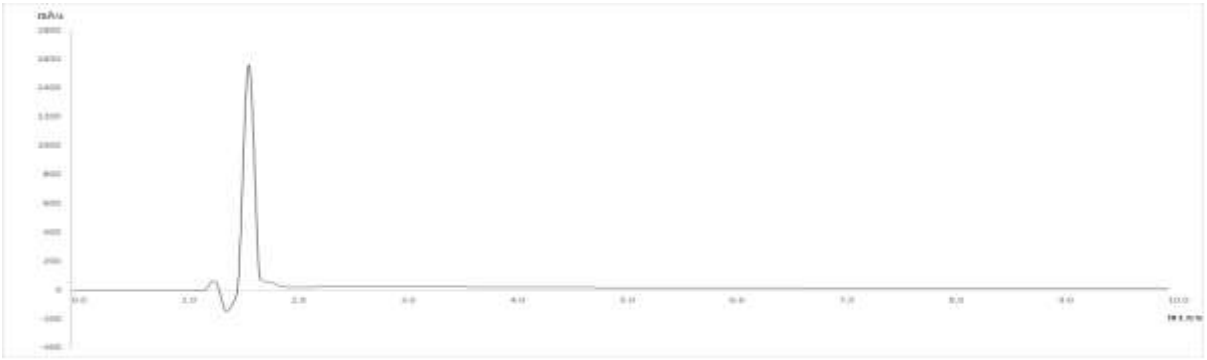

Figure 6: Chromatogram for blank sample

SHIMADZU LC REPORT

SAMPLE: BLANK

FLOW RATE: 10ml/min

INJECTION VOLUME: 20µL

WAVLENGTH: 220nm

RUNTIME:10.0MINS

Optimized Chromatographic conditions

Mobile Phase – 55:45 (10mm Potassium orthophosphate buffer and ACN)
Flow rate 1.00ml/min
Detector Wavelength 220nm
Runtime 10 mins
Retention time
Carbamazepine-3.529 mins
Diclofenac sodium -7.309 mins
Pantoprazole (IS)-4.189 mins

3.2 Validation parameters:

3.2.1 Linearity:

Carbamazepine and Diclofenac both had linear standard curves from 20 ng/mL to 1000 ng/mL. Dividing the peak area of drug with that of IS was used to quantify samples. As can be seen in the picture below, plasma concentrations were plotted against peak area ratios. Linear regression analysis using Carbamazepine yielded a slope of Y=72.599X+229.91 and a correlation value of 0.9997 (Figure 7). For Diclofenac, the slope Y=89.704X+104.34 has a correlation value of 0.9999 (Figure 8).

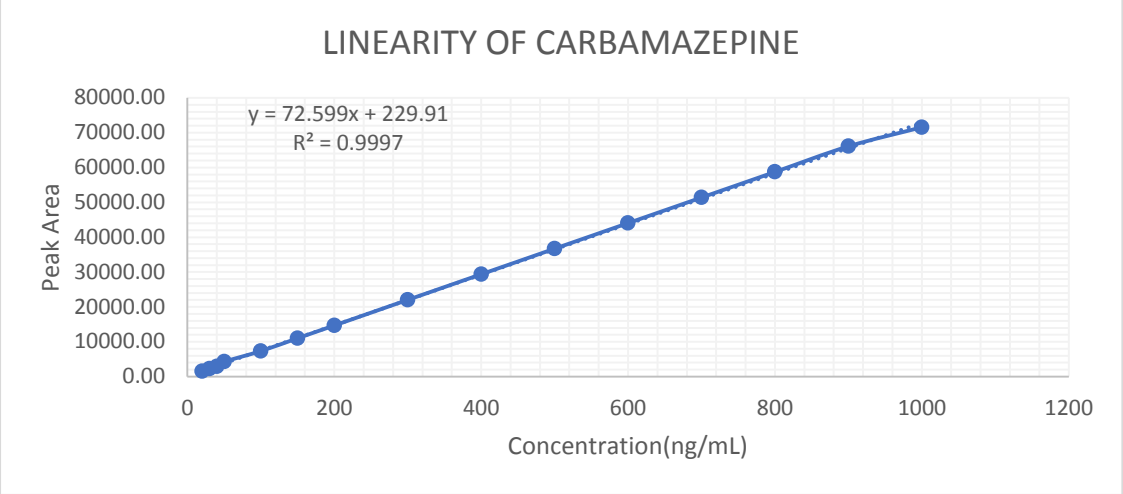

Figure 7: Linearity curve for Carbamazepine

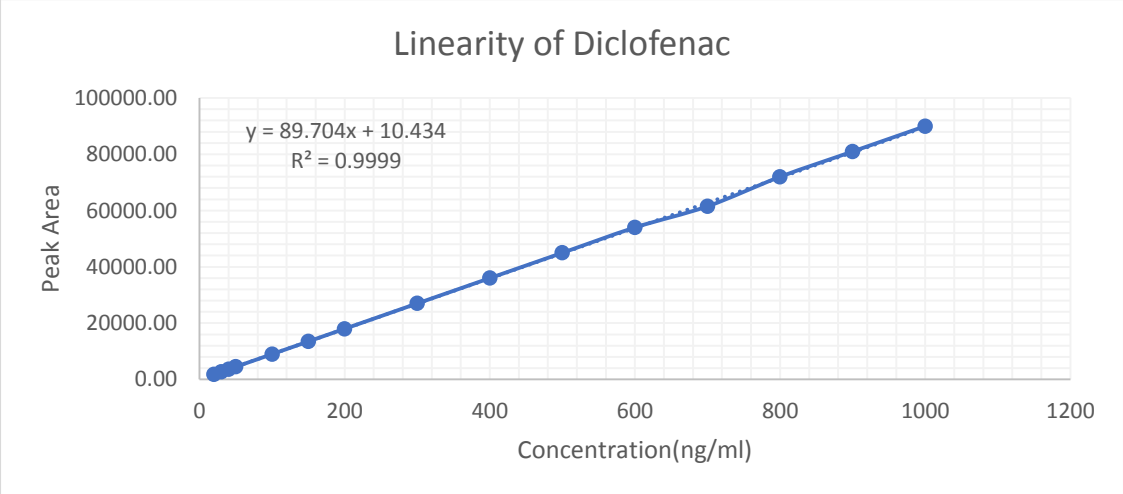

Figure 8: Linearity curve for Diclofenac

3.2.2 Precision of Carbamazepine and Diclofenac

Triplicate determinations of drug samples at concentrations of 70, 550, and 950 ng/mL were used to assess the method's precision and accuracy both within and between days. In this case, precision was reported as a percentage of the RSD. For Carbamazepine, precision ranged from 0.03 to 0.08 percent on an individual day, and from 0.02 to 0.04% over the course of many days. In case of Diclofenac, inter- and intra-day precision ranged between 0.01-0.03 and 0.02, respectively. Carbamazepine and Diclofenac both had RSDs less than 2, proving the precision of the approach.

3.2.3 LOD and LOQ of Carbamazepine and Diclofenac

As measured, the LOD and LOQ for Carbamazepine were 44.85 and 135.91ng/ml, respectively, whereas those for Diclofenac were 13.37 and 40.51ng/ml.

3.2.4 Accuracy of Carbamazepine and Diclofenac:

Table 1 displays the relative standard deviations (RSDs) of the two medications' accuracy values. The technique's accuracy and precision fulfilled the acceptance requirements.

Table 1: Accuracy of Carbamazepine and Diclofenac

	Peak Area Carbamazepine	**Peak area Diclofenac**
Accuracy 650ng/ml	44835.22	58490.90
	47720.77	55441.23
	48546.21	59634.21
	45891.20	60235.11
	40054.66	55997.42
SD	3332.31	2148.07
MEAN	45409.61	57959.77
%RSD	0.07	0.04

3.3 Therapeutic Drug Monitoring of Carbamazepine

TDM for Carbamazepine included a total of 84 patients, including 30 women and 54 males. 47 males and 22 females exhibited serum levels within the therapeutic range (Table 2). Overall, serum concentrations for women averaged 4995.54ng/ml and for males, 3485.92ng/ml. The total dosage range for the study was around 100 - 400 milligrams.

Table 2: TDM of Carbamazepine

PARAMETERS	**MALE**	**FEMALE**
Total Subjects	54	30
Within Therapeutic Range	47	22
Sub-Therapeutic Range	7	8
Age ratio	22-55	20-50
Mean Serum Concentration	4995.54	3485.92
Dose Range	100-400mg	

Between the male and female populations, a static Welsch's t-test was performed ($p \leq 0.05$; $p=0.005$) (Table 3).

Table 3: Welsch's t-Test: Two-Sample Assuming Unequal Variances (P≤0.05)

Parameters	**Variable 1(Male)**	**Variable 2(Female)**
Mean	4955.54	3485.92
Variance	6592720.07	3955766.45
Observations	54	30
Hypothesized Mean Difference	0	
degree of freedom	73	

T_Stat	2.916	
P(T≤t) two-tail	0.005	
T_crit two-tail	1.993	

A one-way anova test was used to compare males and females ($p \leq 0.05$; $p=0.008$). A simple table may be used to summarize the findings of an ANOVA. Table 4 illustrates the format often used, with the symbols SS, Df, and MS standing for Sum of Squares, degrees of freedom, and Mean Square, correspondingly.

Table 4: Summary of ANOVA results

Carbamazepine Anova One Way						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Male	54	267599.3	4955.542	6592720		
Female	30	104577.5	3485.916	3955766		
ANOVA						
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	41653307.39	1	41653307	7.359061	0.008129	3.957388
Within Groups	464131390.8	82	5660139			
Total	505784698.2	83				

Categories such as work (Figure 9), level of education (Figure 10), and marital status (Figure 11) (among men and women) were also tracked as part of the research.

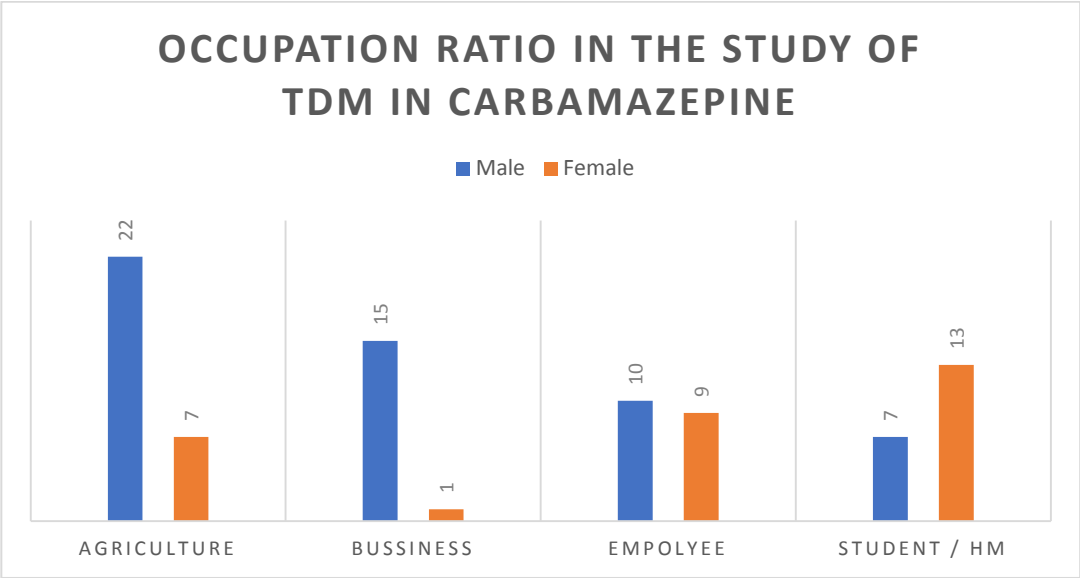

Figure 9: Occupation ratio in the study of TDM in Carbamazepine

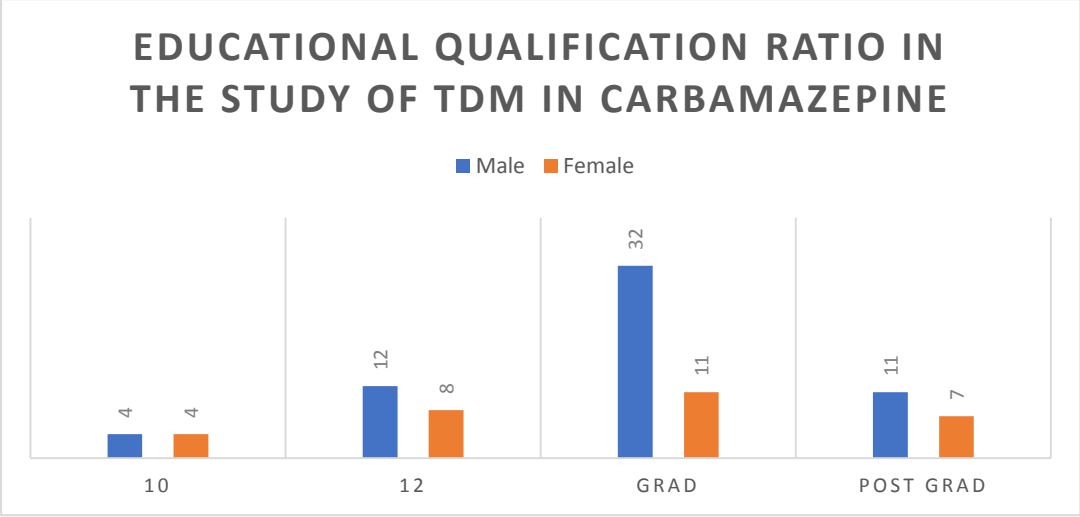

Figure 10: Educational qualification in the study of TDM in Carbamazepine

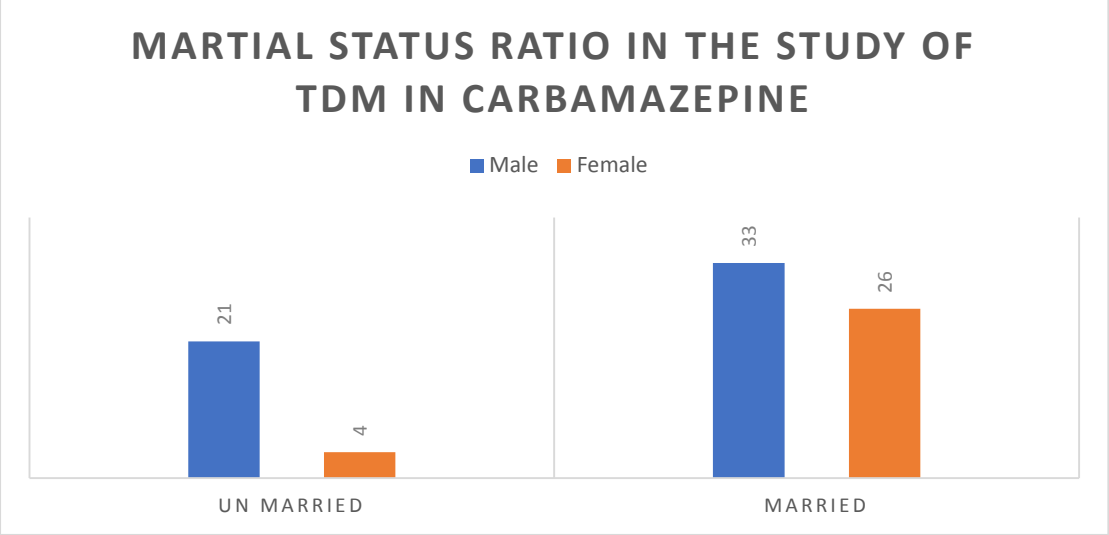

Figure 11: Marital status in the study of TDM in Carbamazepine

4. Discussion

The current approach was efficient, cost-effective, and adaptable to any bioanalytical parameters. Inter-day and intra-day precision for 70, 550, and 950 ng/ml for Carbamazepine were 0.02-0.04, 0.03-0.08, respectively, while for Diclofenac values are 0.01-0.03 and 0.02. All of these values were calculated in accordance with ICH criteria. Carbamazepine has a LOQ of 135.91ng/ml and a LOD of 44.85ng/ml, whereas Diclofenac has LOQ of 40.51ng/ml and LOD of 13.37ng/ml. Carbamazepine TDM demonstrated remarkable outcomes, with the average serum levels for 84 participants reaching steady state after receiving treatment for a long time. The mean serum levels for females was 4995.54ng/ml, whereas the mean serum concentration for males was 3485.92ng/ml. The total dosage range for the study was around 100 - 400 milligrams.

5. Conclusion

In summary, the suggested technique for measuring Carbamazepine concentration was quick, repeatable, and accurate. Consequently, RCTs in a wider population are anticipated, ideally with a broad dosage (100-400mg) to correspond with the overall serum levels of the population as well as multiple comedication for dose modifications to achieve the maximal safe dosage in participant. Such studies verify the correlation between Carbamazepine concentration and clinical response, providing crucial information for determining the drug's effectiveness according to the specific needs of each individual patient.

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