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Research article

Validated UFLC bioanalytical method for simultaneous estimation of Carbamazepine and Olanzapine: an interaction study

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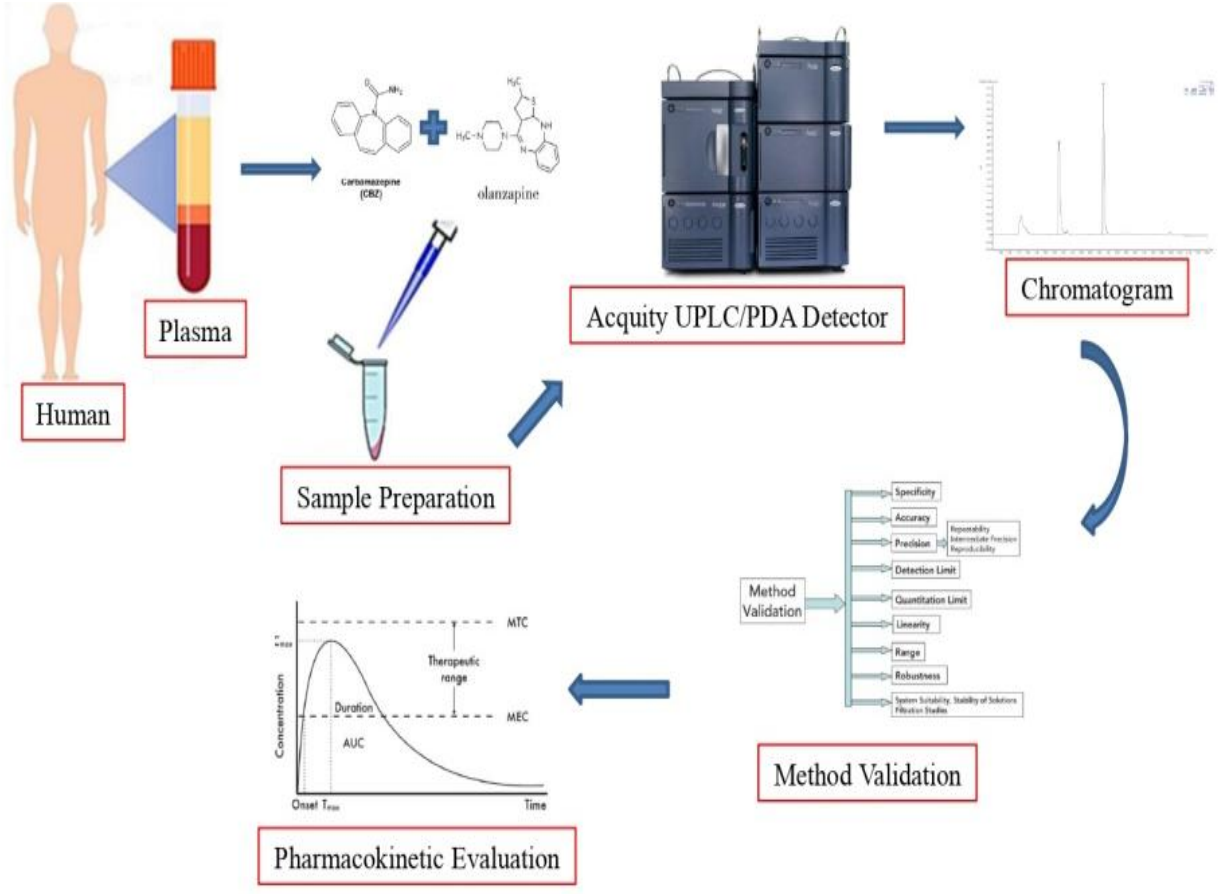
ABSTRACT

Epilepsy is a pervasive and chronic neurodegenerative condition. The most commonly prescribed medications are the carbamazepine and olanzapine. This study is aimed to establish a bio-analytical method and validate for concurrent assessment of Carbamazepine and Olanzapine as per USFDA guidelines.



The chromatographic segregation was executed by using Accucore C18, (50 x 4.6mm, 2.6µ) column by applying gradient elution using formic acid (0.1%) in aqueous phase (A) and methanol (B) following gradient: initial 10% B is maintained for 1 minute, 1 to 6minutes (5% A, 95% B), 6 to 9.5 minutes (90%A, 10% B) and maintained with 10% B for 2.5 minutes, detection was carried at the 270 nm by employing 0.4 mL/min flow rate. The separation of drugs from the human plasma was achieved using protein precipitation technique.

The analytes exhibited 5.34 min and 7.86 min retention time for olanzapine and carbamazepine respectively. The developed method has the linearity range of 50-250µg/ml and 20-100µg/ml for carbamazepine and olanzapine respectively and the regression co-efficient (r 2) was 0.9 for the both drugs. Robustness and injectable reproducibility was found to be within the limit and the results were reproducible.

Based on these results, conclude that the developed method was precise and accurate for the simultaneous estimation of carbamazepine and olanzapine. This method can be adopted for simultaneous estimation of drugs and will be beneficial for in-vivo studies & pharmacokinetics studies.

Keywords: Carbamazepine and olanzapine, Human Plasma, Validation, Ultra Performance Liquid Chromatography.

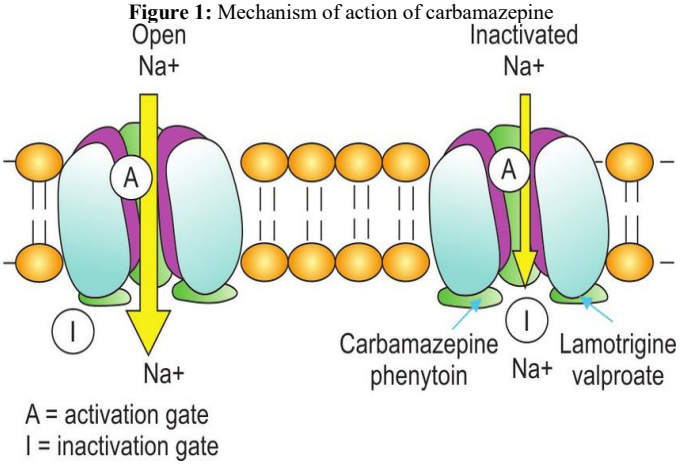
INTRODUCTION

Epilepsy is prevalent, disabling condition with an occurrence with roughly 50 new cases reported per 100,000 population each year. Epilepsy characteristics by recurrent, unapproved seizures. One of the main causes of epilepsy is brain dysfunction. Around 1% of population abides from epilepsy and around 1/3rd of them have refractory epilepsy i.e., the seizures remain uncontrolled despite treatment with two or more anti-epileptic drugs. Epilepsy is diagnosed by various methods such as Electroencephalogram (EEC), neuro imaging like CT scan, MRI scan PET scan and metabolic evaluation, genetic testing [1]. Recent studies indicate that nearly 90% of the 70 million individuals affected by epilepsy globally live in developing countries. Advances in genetic testing have significantly enhanced the ability to identify the underlying causes of different forms of epilepsy. However, applying these tools effectively remains a complex task that demands prior clinical experience and specialized knowledge [2]. In clinical practice, the anticonvulsant carbamazepine (CBZ) is prescribed as the first-line therapy for generalized tonic-clonic and partial seizures [3]. Over the past year, clinical trial results have demonstrated the effectiveness of low-cost interventions that have the potential to benefit large populations of people with epilepsy, particularly in low-income countries [4].

The last ten years have seen an increase in the usage of carbamazepine to treat a variety of mood and anxiety disorders. After oral administration, carbamazepine is quickly absorbed. Cmax is attained between 2 and 6 hours after administration, with significant individual variability. In addition to potentially inducing its own metabolism, carbamazepine is metabolized in the liver [5]. Carbamazepine is totally metabolized and small traces are excreted unchanged in the urine [6].

Gamma amino butyric acid (GABA), an inhibitory neurotransmitter, is upregulated by carbamazepine, whereas glutamate (Glue), an stimulatory neurotransmitter, is downregulated. The inactivated sodium channels are targeted by carbamazepine, which delays their reactivation. In addition, it blocks calcium influx across the synaptic membrane [7]. The mechanism of action of carbamazepine is shown in Figure1.

Figure 1: Mechanism of action of carbamazepine



Literature survey revealed that few Spectrophotometric methods and High-Performance Liquid Chromatography (RP-HPLC), Liquid Chromatography-Mass Spectrometry (LC-MS/MS) methods have been reported for the estimation of Carbamazepine with other drugs.

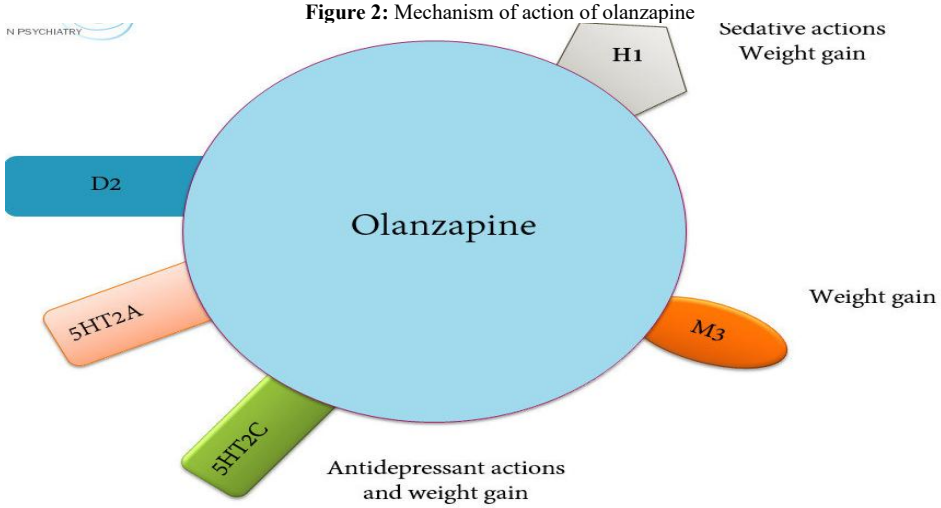
Olanzapine shows superior performance with well-controlled trials in patients with schizophrenia or related psychoses in terms of significant progress on as evidenced by improvements in psychopathology rating scales, while its effects on positive psychotic symptoms were comparable [8].

Olanzapine, that predominantly affects dopamine and serotonin receptors, belong to the class of second-generation atypical antipsychotic. By inhibiting dopamine D2 receptors, it functions as an antagonist on the mesolimbic pathway, preventing dopamine from activating post-synaptic receptors. Olanzapine's easy dissociation due to its loose binding promotes regular dopamine neurotransmission. Olanzapine's action on serotonin reduces negative symptoms such anhedonia, flat affect, alogia, avolition, and inattentiveness [9] Shown in Figure 2.

Bioanalytical method is a collection of steps used to gather process, store, and to determine the concentration of the analyte in a biological matrix. Bioanalysis is an ever-evolving field with

significant potential for advancements in sensitivity, specificity, accuracy, efficiency, throughput, data quality, and environmental sustainability. In forensic and clinical toxicology, the accuracy of results is crucial since it is obviously necessary for the proper interpretation of toxicological results [10]. Bioanalytical methodologies have been reported that facilitate the efficient extraction and sensitive detection of olanzapine, considering both traditional and innovative sample pretreatment strategies along with their respective advantages and limitations [11].

Figure 2: Mechanism of action of olanzapine



In a recent investigation, a sensitive and selective GC–MS method was established for therapeutic drug monitoring (TDM) of total and free carbamazepine, along with its epoxide metabolite, in human plasma. [12].

A recent survey revealed that a variety of analytical techniques have been employed for the estimation of olanzapine (OLZ) in both bulk and solid dosage forms. These include UV spectrophotometry, mass spectrometry (MS), liquid chromatography with tandem mass spectrometry (LC-MS/MS), and chromatographic methods such as high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) [13].

Based on literature report we observed, only limited work has been reported. Normally estimation of carbamazepine and olanzapine has been reported along with other drugs or alone by analytical method but lack in simultaneous estimation of carbamazepine and olanzapine by bio analytical studies.

Interaction studies of carbamazepine and olanzapine

CYP3A4 and CYP1A2 are just two of the P450 cytochromes that carbamazepine has been demonstrated to stimulate. Given that CYP1A2 is a key factor in the biotransformation of olanzapine, the interaction can be explained by carbamazepine's stimulation of CYP1A2, which boosts both the metabolic pathways of olanzapine. This interaction is regarded as clinically insignificant as olanzapine exhibits a broad therapeutic index, and the alterations in plasma concentration remain within the fourfold interpatient variability range observed under normal clinical conditions, posing no safety risk [14].

MATERIALS AND METHODS

Materials

The chemicals, reagents, and solvents utilized for method development and validation are detailed as follows in Table 1.

Table 1: List of chemicals, reagents and solvents

Name of chemicals / reagents	Manufacturer
Methanol (HPLC quality solvent)	S D Fine chem. Pvt. Ltd., Mumbai.
Formic Acid (A R grade)	Rankem Chemicals, Mumbai.
Dimethylsulphoxide (L R quality solvent)	S D Fine chem Pvt. Ltd., Mumbai.
Millipore water (HPLC grade)	In house.

Analytical instrumentation

The apparatus and instruments employed in the bioanalytical method development and validation are presented in Table 2.

Table 2: Outline of instruments & equipment

Instruments/equipment	Mode/maker
UPLC, PDA detector (Acquity H-class)	Waters Corporation, USA
Automatic sampler (Serial # C10UPA554M)	Waters Corporation, Singapore
Ultra-performance binary solvent manager (Serial # C10UPB081A)	Waters Corporation, Singapore
Centrifuge (CAT-1010)	Tarsons, India.
C18, 50 x 4.6mm, 2.6µ	Accucore
Electronic analytical balance	Mettler Toledo, India
Micropipettes	Eppendorf AG, Germany.
Vortex mixer	Grant Bio, UK.
Sonicator	Oscar Ultrasonic, India.

Preparation of mobile phase

The mobile phase comprised of 0.1% formic acid in water (A) and methanol (B). It was subsequently filtered through a 0.45 µm membrane filter and then sonicated for 20 minutes to remove dissolved gases and ensure homogeneity.

Preparations of standard solutions

Carbamazepine (CBZ)

Carbamazepine (0.1g) was dissolved in DMSO solution (10ml) and was sonicated for 10 minutes (A). Further dilution was made by pipetting 1ml of A in 10ml of methanol (B). The serial dilutions were prepared from stock solution B in methanol to obtain 50-250 µg/ml concentration of carbamazepine.

Olanzapine (OZP)

Olanzapine (0.1g) was dissolved in DMSO (10ml) and was sonicated for 10 minutes (A). Further dilution was made by pipetting 1ml of A in 10ml of methanol (B). The serial dilutions were prepared from stock solution B in methanol to obtain 20-100 µg/ml concentration of Olanzepine.

Bio-analytical method development and validation

Preparation of plasma samples

Carbamazepine (CBZ) and Olanzapine (OZP)

To extract the medication (CBZ and OZP) from human plasma, a straightforward protein precipitation technique was employed. Using a Vortex mixer, 0.5 ml of sample solution, 1 ml of plasma, and 0.5 ml of methanol were added to a 2 ml aliquot and stirred for 5 minutes. For fifteen minutes, the sample solution was centrifuged. After being moved to a different tube, the supernatant was carefully filtered through a 0.2 µm syringe filter.

Preparation of quality control samples

Carbamazepine (CBZ)

The quality control samples of 50 (LQC), 150 (MQC), and 250 (HQC) µg/ml were prepared by adding 1ml of blank plasma.

Olanzapine (OZP)

The quality control samples of 20 (LQC), 60 (MQC), and 100 (HQC) µg/ml were produced by spiking 1 ml of blank plasma with the aforementioned drug solutions.

Validation of developed method

Selectivity and Specificity

The process was assessed by differentiating and quantifying the analyte amidst other components in the plasma sample. Analysis of blank human plasma samples was conducted using a minimum of six sources. All blank samples were evaluated considering possible overlap, and the selectivity was ascertained at the LLOQ.

Accuracy, Precision and Recovery

The accuracy was evaluated by repeated analysis of QC samples spiked with known concentrations of the analyte. Each concentration was tested using a minimum of five replicates. It was agreed that at least three doses within the anticipated range of study sample concentrations were acceptable. When the process was repeated on several aliquots of single homogenous plasma, the precision was achieved by the proximity of individual measurements of an analyte. Each concentration was tested using a minimum of five determinations. It was advised to include at least three concentrations within the range of anticipated study sample values. There were two types of precision: within-run and between-run. A single analytical run's precision was evaluated using within-run precision. An evaluation of precision across time, between-run precision may entail various analysts, tools, reagents, and labs

Recovery of the analyte was determined by comparing the detector response of standard solutions with that of plasma samples spiked and extracted under identical conditions. The USFDA publication states that analyte recovery does not have to be 100%. The analyte recovery was evaluated by extracting samples at three concentrations (low, medium, and high) and comparing them with unextracted standards.

Calibration curve

Plotting the drug sample's peak region in blank human plasma allowed for the creation of the six-point calibration curve. The data were fitted to linear regression analysis after various dilution factors were evaluated. The regression correlation coefficient (r2) of 0.999 or higher was required for the calibration curve. With the exception of LLOQ, which was set at ±20%, The acceptability criteria for each back-calculated standard concentration were set at ±15% deviation from the nominal value.

Stability experiments

Stability tests were performed, Using LQC, MQC, and HQC samples, to assess the drug's stability in plasma samples under various circumstances.

Stock solution stability

Room temperature stability of the stock solutions, both original and spiked, was assessed, and the analytes remained stable throughout the study period of six hours. Following that, the samples underwent analysis, processing, and comparison.

Autosampler stability

The stability was assessed by maintaining the original and spiked solutions at room temperature for twenty-four hours. Following that, the samples underwent analysis, processing, and comparison with the recently made solutions.

Short-term stability

Bench-top stability was evaluated by keeping both the original and the spiked stock solution at ambient temperature for 12 hours. Following that, the samples underwent analysis, processing, and comparison with the recently made solutions.

Freeze–thaw stability

To determine freeze-thaw stability, the stock solution and spiked stock solution were frozen at -20^0C and thawed at room temperature until the three cycles were finished. Following that, the samples underwent analysis, processing, and comparison with the recently made solutions.

Freezer stability

To assess freezer stability, the original and spiked stock solutions were stored at −20 ± 1 °C for 25 days. Following that, the samples underwent analysis, processing, and comparison with the recently made solutions.

If the test results fell between the established acceptance criteria of accuracy and precision, aforementioned treated samples were deemed stable.

RESULT

Bio analytical method development and validation

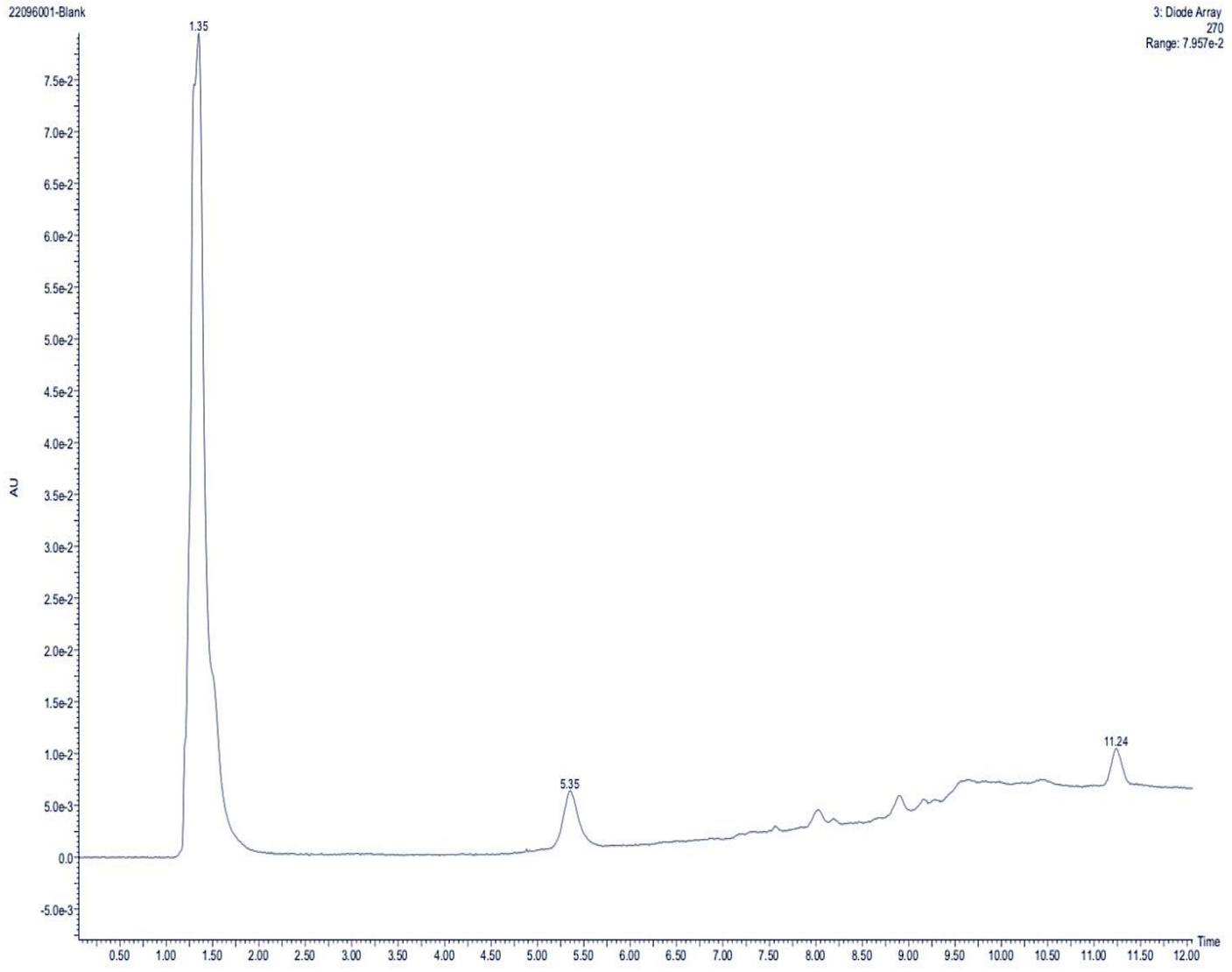
Chromatographic method development and optimization

The Carbamazepine and Olanzapine was eluted on a C18 (50 x 4.6mm, 2.6µ) column. Mobile phase A was prepared with formic acid (0.1%) in distilled water, while mobile phase B comprised of methanol. Chromatographic separation was achieved by pumping the mobile phase through the column at a constant flow rate of 0.4 mL/min under optimized conditions using the following gradient program: to start with 10% B is maintained for 1 minute, 1 to 6minutes (10 to 95% B), held for 3 minutes, 9 to 9.5 minutes (90 to 10% B) and maintained at 10% for 2.5 minutes.1ml/min. Detection were done at 270 nm, the time of elution was 5.34 min and 7.86 min for olanzapine and carbamazepine respectively showed in the below Figure 3, 4 & 5.

Table 3: Standard chromatographic conditions for olanzapine and carbamazepine

Instrument	Ultra-Performance liquid chromatograph (UPLC) Acquity H-class, Waters
Column	C18 Accucore (50 x 4.6mm, 2.6µ)
Mobile phase	Water (0.1% Formic acid) and meoh
Wavelength	270 nm
Flow rate	0.4 ml/min
Elution Time	3.75 min and 6.17 min for OZP &CBZ.
Run Time	12 min

Figure 3: Chromatogram of Blank Plasma



Method Development

Figure 4: Chromatogram of Olanzapine and Carbamazepine

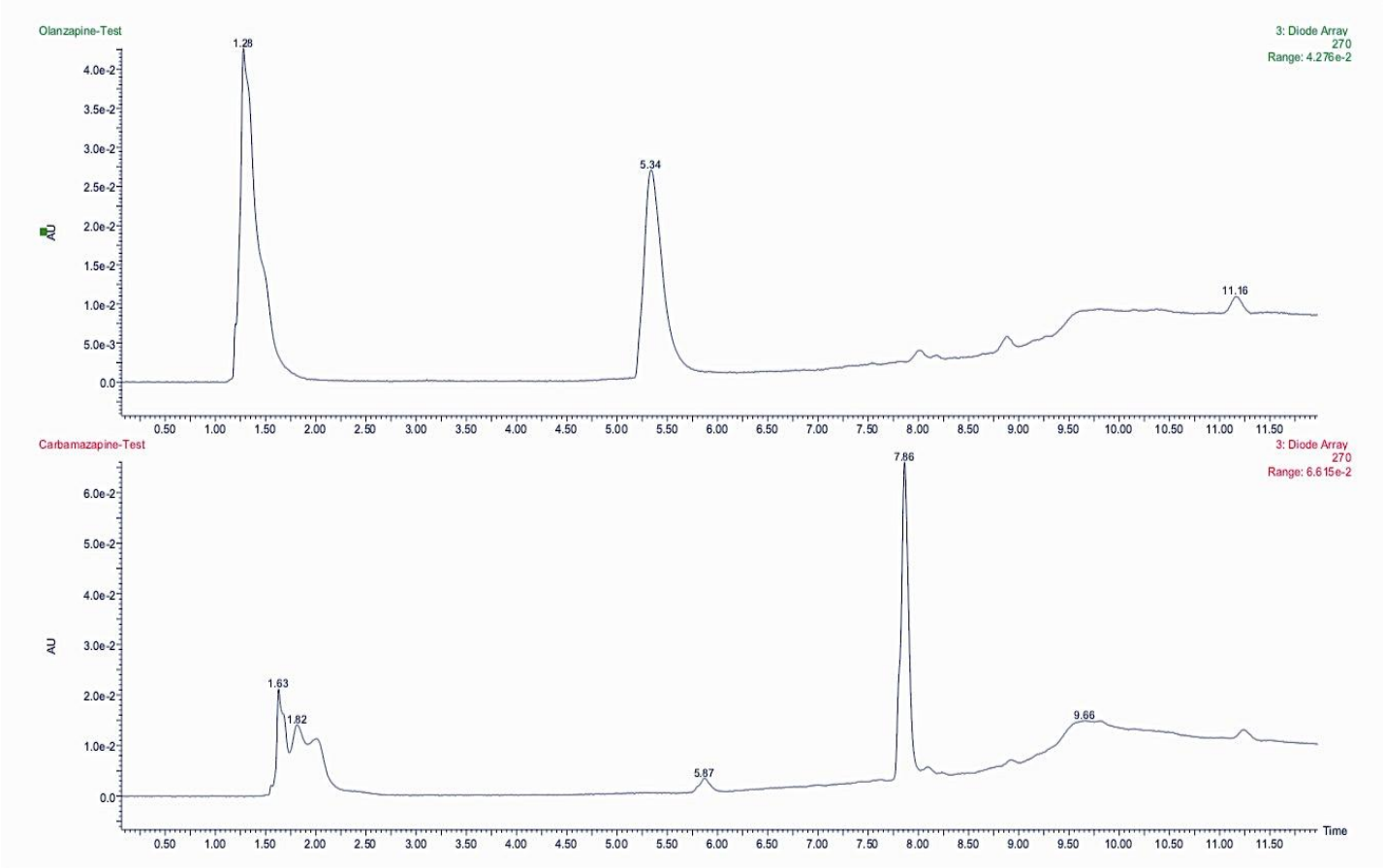
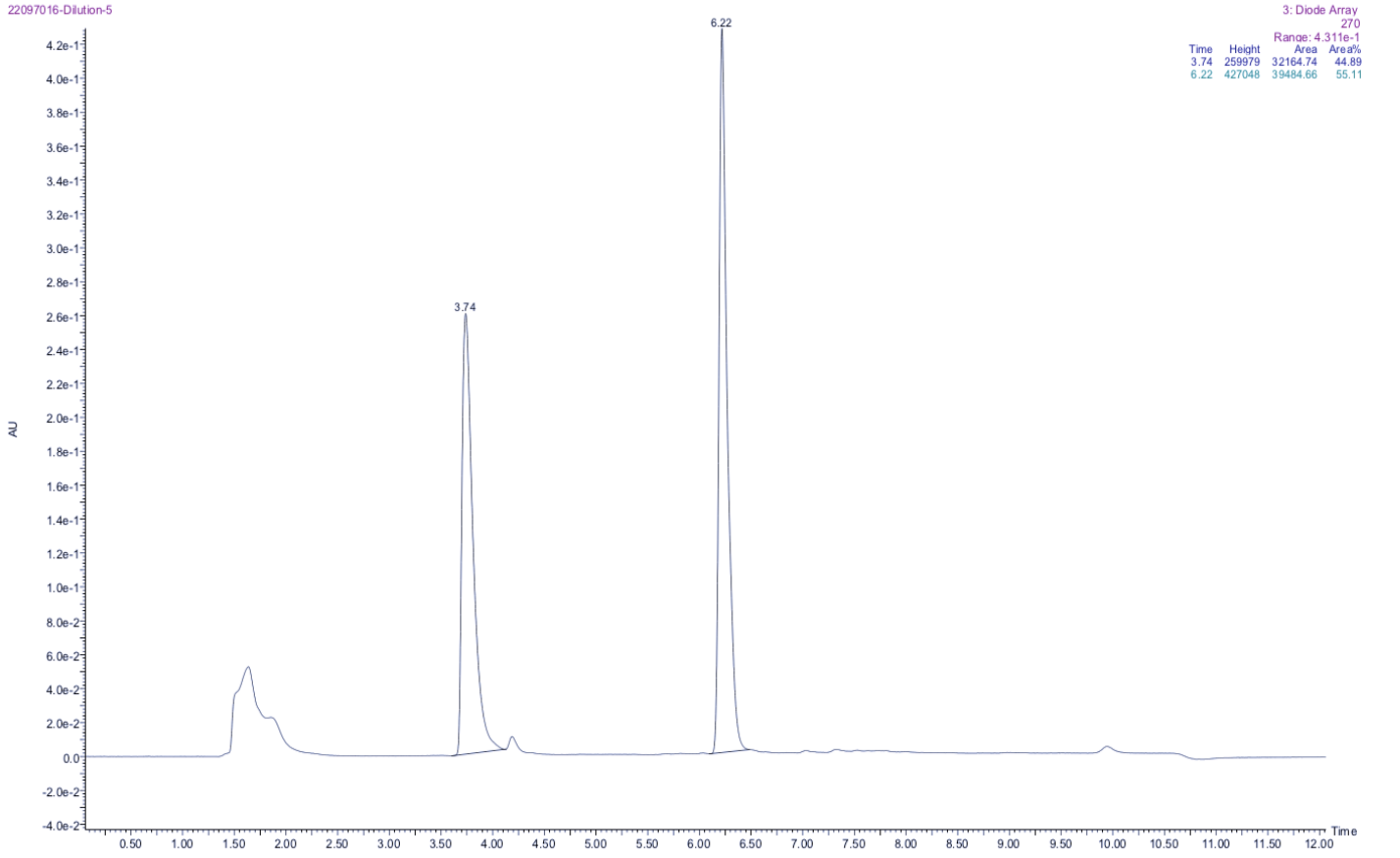


Figure 5: Chromatogram of OZP and CBZ with Plasma (100/250) µg/ml



Analytical method validation as per USFDA guidelines
Linearity and standard calibration curve
Olanzapine

Calibration was evaluated in the range of 20–100 µg/ml by constructing a calibration curve, which was then assessed using its correlation coefficient to confirm the proportional relationship between concentration and analytical response. The correlation coefficient (r^2) for the entire calibration curve was found to be 0.993 represented in Figure 6.

Carbamazepine

Linear calibration was assessed in the concentration series of 50–250 µg/ml by constructing a calibration curve, and the degree of correlation between concentration and response was determined by evaluating the curve's correlation coefficient. The correlation coefficient (r^2) for the entire calibration curve was found to be 0.995 represented in Figure 7.

Method sensitivity and specificity

Olanzapine and carbamazepine at concentrations of 20 µg/ml and 50 µg/ml were readily identified from human plasma after a 12-minute run period. It would be easy to confirm the presence of olanzapine and carbamazepine by comparing the retention time of the sample with that of a reference medication. After analyzing every chromatogram, it was discovered that endogenous substances had no effect on olanzapine and carbamazepine.

Selectivity

No notable contamination or interference from substances naturally present in the sample was detected and represented in Figure 8 & 9.

Figure 6: Standard Calibration Graph of Olanzapine

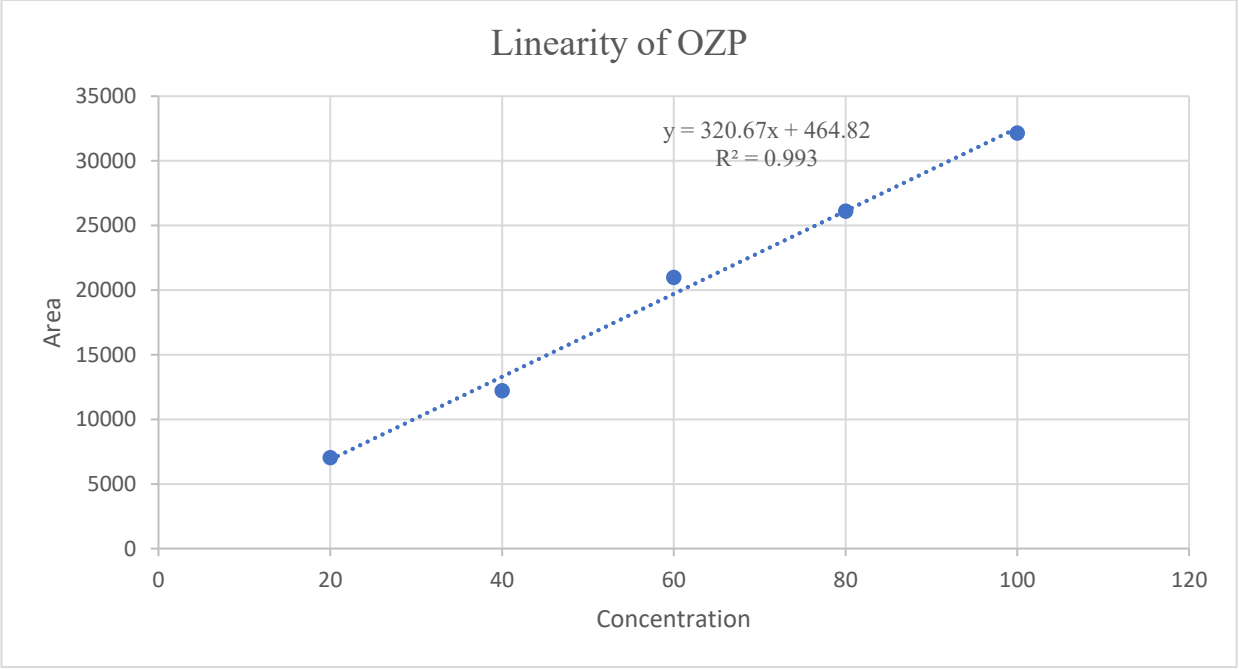


Figure 7: Standard Calibration Graph of Carbamazepine

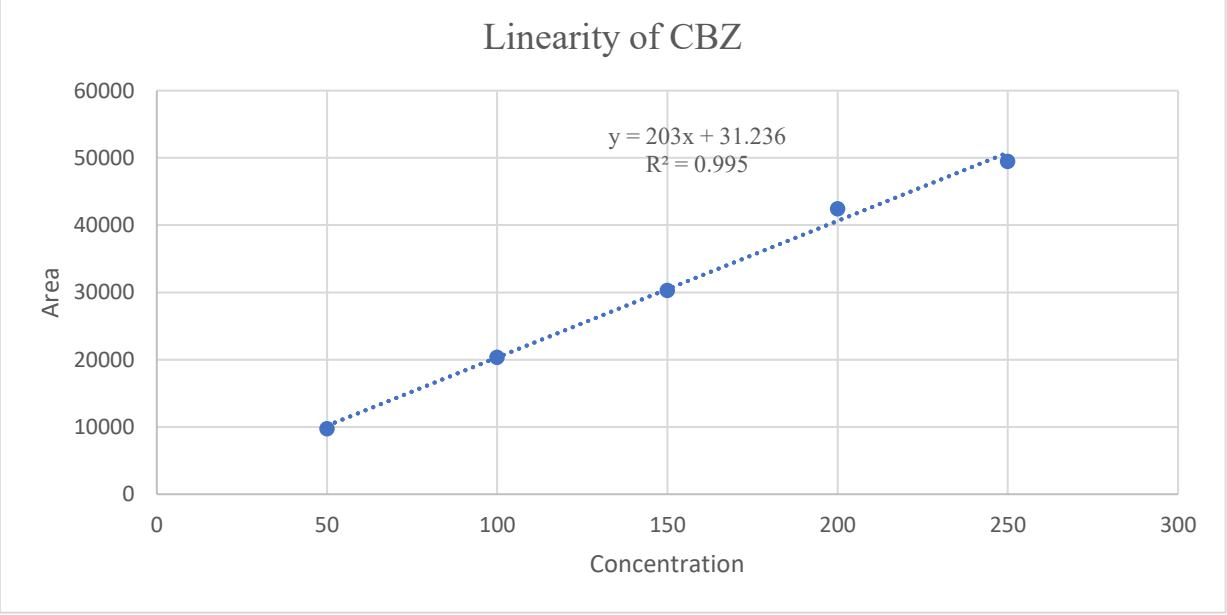


Figure 8: Selectivity Chromatogram of Blank Plasma

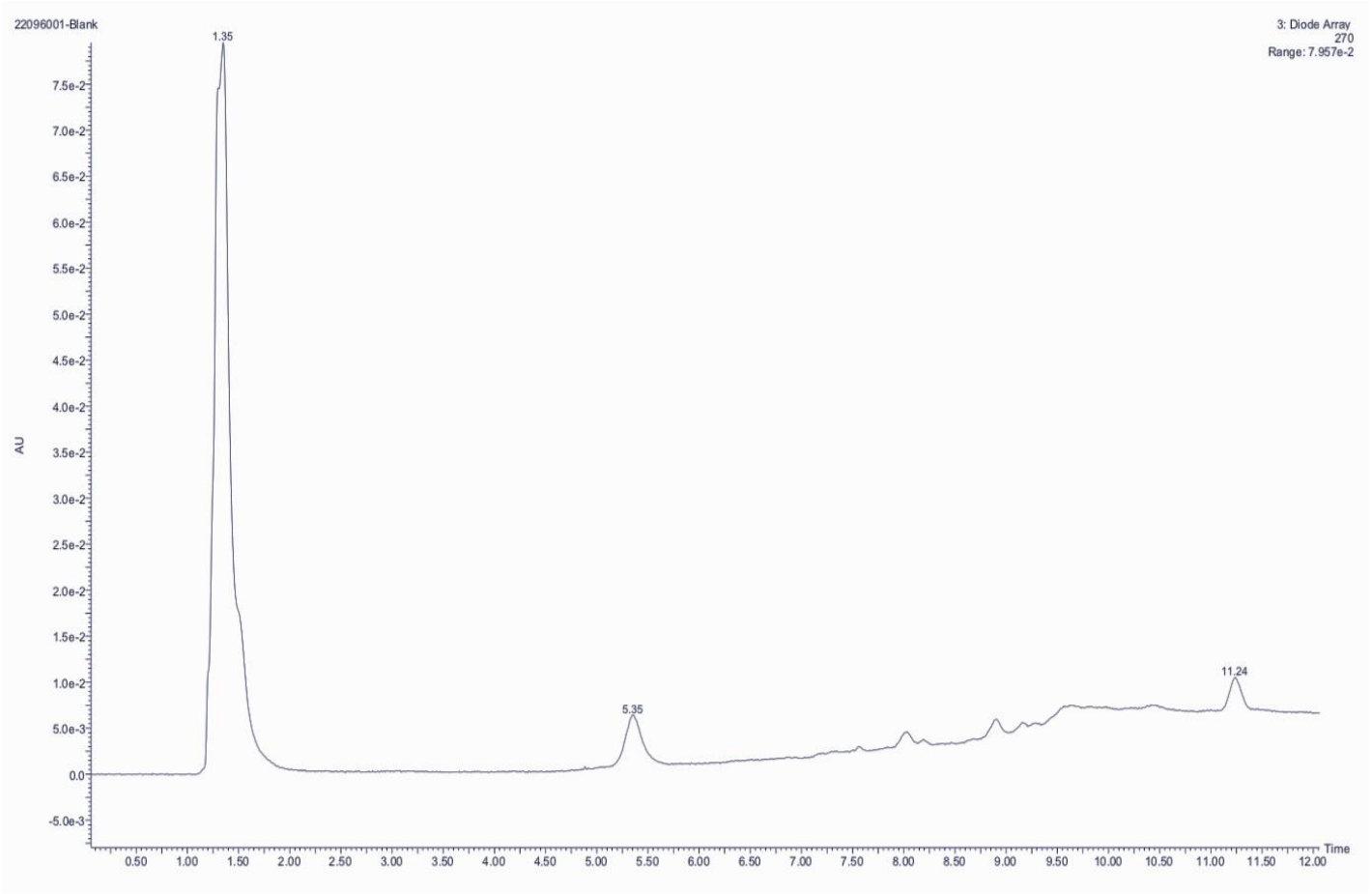
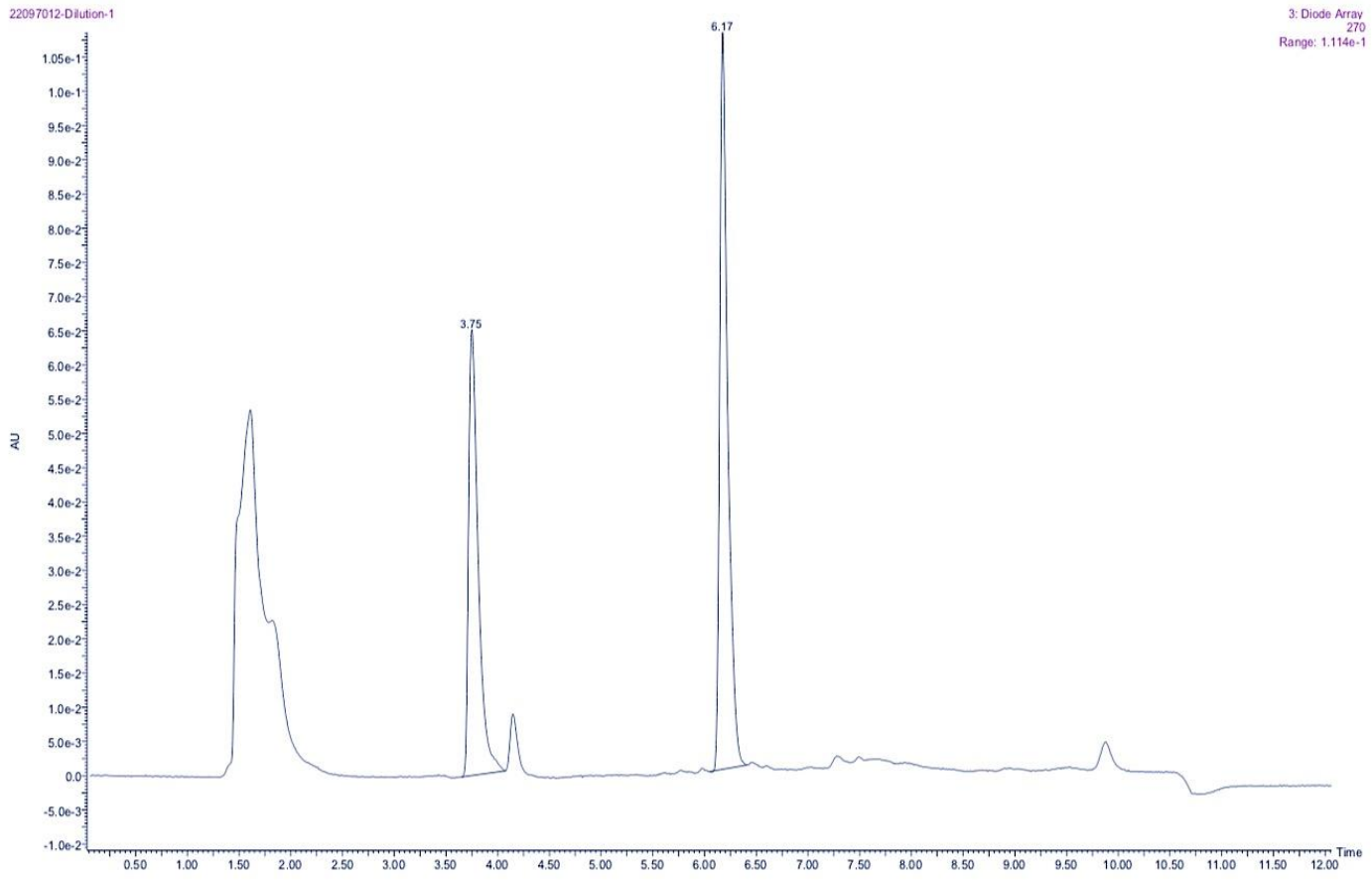


Figure 9: Selectivity Chromatogram of OZP and CBZ with Plasma



Precision

Intraday Precision

Table 4: Intra-Interday precision data of olanzapine by RP-UPLC method

Morning

QC Levels	Conc. µg/ml	Peak area	Quantified conc. µg/ml	Average	STD DEV	% CV
LQC	20	7039.107 7041.128 7039.312	20.14 20.95 20.00	20.363	0.512	2.514
MQC	60	21978.627 21978.832 21977.270	60.00 60.23 59.89	60.373	0.743931	1.230
HQC	100	32164.744 32165.467 32164.124	100.02 100.76 99.98	100.586	1.016333	1.01

Evening

QC Levels	Conc. µg/ml	Peak area	Quantified conc. µg/ml	Average	STD DEV	% CV
LQC	20	7039.378 7039.104 7039.786	20.10 19.99 20.53	20.206	0.285	1.41
MQC	60	21978.876 21978.624 21979.436	60.10 61.01 60.87	60.66	0.49	0.807
HQC	100	32164.944 32164.832 32164.457	100.12 101.56 99.96	100.546	0.881211	0.876

Interday Precision (Day 1)

QC Levels	Conc. µg/ml	Peak area	Quantified conc. µg/ml	Average	STD DEV	% CV
LQC	20	7039.521 7038.690 7039.312	20.14 19.95 20.00	20.03	0.51	2.5
MQC	60	21978.624 21978.636 21979.472	60.00 60.01 60.98	60.33	0.56	0.9
HQC	100	32164.948 32164.744 32164.122	100.01 99.9 100.2	100.03	0.1	1.15

Day 2

QC Levels	Conc. µg/ml	Peak area	Quantified conc. µg/ml	Average	STD DEV	% CV
LQC	20	7039.415 7039.319 7039.312	20.11 20.01 20.8	20.30	0.43015	2.118
MQC	60	21978.814 21978.724 21977.984	60.10 61.06 59.97	60.37	0.59534	0.985
HQC	100	32164.874 32164.697 32164.456	100.11 101.7 99.95	100.58	0.96748	0.961

Day 3

QC Levels	Conc. µg/ml	Peak area	Quantified conc. µg/ml	Average	STD DEV	% CV
LQC	20	7039.910 7039.123 7039.319	20.1 19.3 21.01	20.136	0.85558	4.249
MQC	60	21978.924 21978.629 21978.220	61.86 60.01 59.98	60.616	1.07686	1.776
HQC	100	32164.744 32164.744 32164.744	100.12 101.09 99.6	100.27	0.75624	0.754

Accuracy

To evaluate accuracy, a known quantity of the standard solution was spiked into pre-analyzed samples (50%, 100%, and 150%). The percentage recovery for olanzapine and carbamazepine was calculated for three concentrations. For all analyses, the percent RSD values were below 2% confirming that no impurities affect and developed analytical method found to be highly accurate as represented in Table 5 and Table 6.

Stability

The plasma sample's stability during various stability periods and storage settings was examined. Six replicates were used for each of the concentration levels (low, medium, and high). The stability of each analyte was determined by evaluating the mean concentrations of each analyte in the stored samples with the corresponding concentrations in freshly prepared QC samples, the % stability was computed. The results, which showed, all the analyte exhibited a

satisfactory stability when subjected to, as demonstrated in Table 7 and 8.

Recovery of drugs from plasma

The recovery of the analyte should be performed in order to confirm the efficiency and reproducibility of the extraction. Recovery studies were carried out by comparing the analytical responses of extracted samples against standard solutions at equivalent concentrations corresponding extracts of blanks spiked with the analyte post-extraction. A summary of the drug recoveries is presented in Table 9 and 10.

Robustness

The robustness of an analytical method assesses its capacity to withstand minor variations in experimental conditions without affecting performance during routine analysis like minor changes in pH, mobile phase composition, temperature, etc. The robustness for this method was successfully validated by optimizing the pH of the mobile phase. The results are shown in Figure10 and 11.

Injection repeatability

Injection reproducibility was evaluated by performing multiple replicate injections in case of instrument failure. The Injection Reproducibility for 3µl and 6µl are shown in **Figure 12 and 13** respectively.

Table 5: Accuracy data for Olanzapine by RP UPLC method (% recovery data)

Drug spiked (%)	STD drug (µg/ml)	Sample (µg/ml)	Total Drug content (n=3)	Drug quantified	% Recovery	Mean	%SD	%RSD
50%	100	50	150	150	100	99.99	0.6650	0.665
				149	99.33			
				151	100.66			
100%	100	100	200	200	100	101.16	1.2583	1.243
				202	101			
				205	102.5			
150%	100	150	250	250	100	99.6	0.6928	0.694
				247	98.8			
				250	100			

Table 6: Accuracy data for Carbamazepine by RP UPLC method (% recovery data)

Drug spiked (%)	STD drug (µg/ml)	Sample (µg/ml)	Total Drug content (n=3)	Drug quantified	% Recovery	Mean	%SD	%RSD
50	40	20	60	59	98.33	99.44	0.964	0.969
				60	100			
				60	100			
100	40	40	80	78	97.5	99.16	1.443	1.455
				80	100			
				80	100			
150	40	60	100	100	100	99.33	1.154	1.161
				98	98			
				100	100			

Table 7: Stability of olanzapine in plasma

Stability	QC sample (spiked) Conc.(µg/ml)	Mean ± SD (µg/ml)	Percent Recovery (%)	RSD (%)
Long-term (30 days)	20	20.33±0.57	101.66	2.838
	60	60.33±1.52	100.55	2.531
	100	101.33±3.21	101.33	3.171
Short-term (8 hours)	20	20.33±1.15	101.66	5.676
	60	60.33±3.21	100.55	5.327
	100	99±3.6	99	3.641
Auto-sampler (24 hours)	20	20.33±0.57	101.66	2.838
	60	61±1.73	101.66	2.839
	100	100.33±2.51	100.33	2.507
Freeze-Thaw	20	20.33±0.57	101.66	2.837
	60	59.33±1.52	98.88	2.573
	100	100.66±3.21	100.66	3.192

Table 8: Stability of carbamazepine in plasma

Stability	QC sample Conc. (µg/ml)	Mean ± SD (µg/ml)	Recovery (%)	RSD (%)
Long-term (30 days)	50	50 ±1	100	2.0
	150	150.33 ±1.5275	100.22	1.0517
	250	250.33±1.5287	100.132	0.6161
Short-term (8 hours)	50	51±1	102	1.9607
	150	151.333±1.5275	100.88	1.0093
	250	250.666±2.0816	100.264	0.8302
Auto-sampler (24 hours)	50	49.6666±1.5725	99.33	2.9703
	150	151±1	100.66	1.0635
	250	250.333±1.57	100.132	0.6099
Freeze-Thaw	50	50.33±1.672	100.89	2.2358
	150	150.33±1.52	100.03	1.08261
	250	251.33±2.086	100.532	0.8301

Table 9: Extraction recovery and matrix effect of olanzapine

QC Levels	In Plasma	Average In Plasma	Recovery %
LQC (20µg/ml)	19 17 16	18	90
MQC (60µg/ml)	52 56 59	55	91.66
HQC (100µg/ml)	94 93 97	94.66	94.66

Table 10: Extraction recovery and matrix effect of carbamazepine

QC Levels	In Plasma	Average In Plasma	Recovery %
LQC (50µg/ml)	47 49 50	47	94
MQC (150µg/ml)	149 150 148	149	99.33
HQC (250µg/ml)	246 249 247	247.33	98.93

Figure 10: Robustness for Mobile phase- A: 0.05% Formic acid in Water

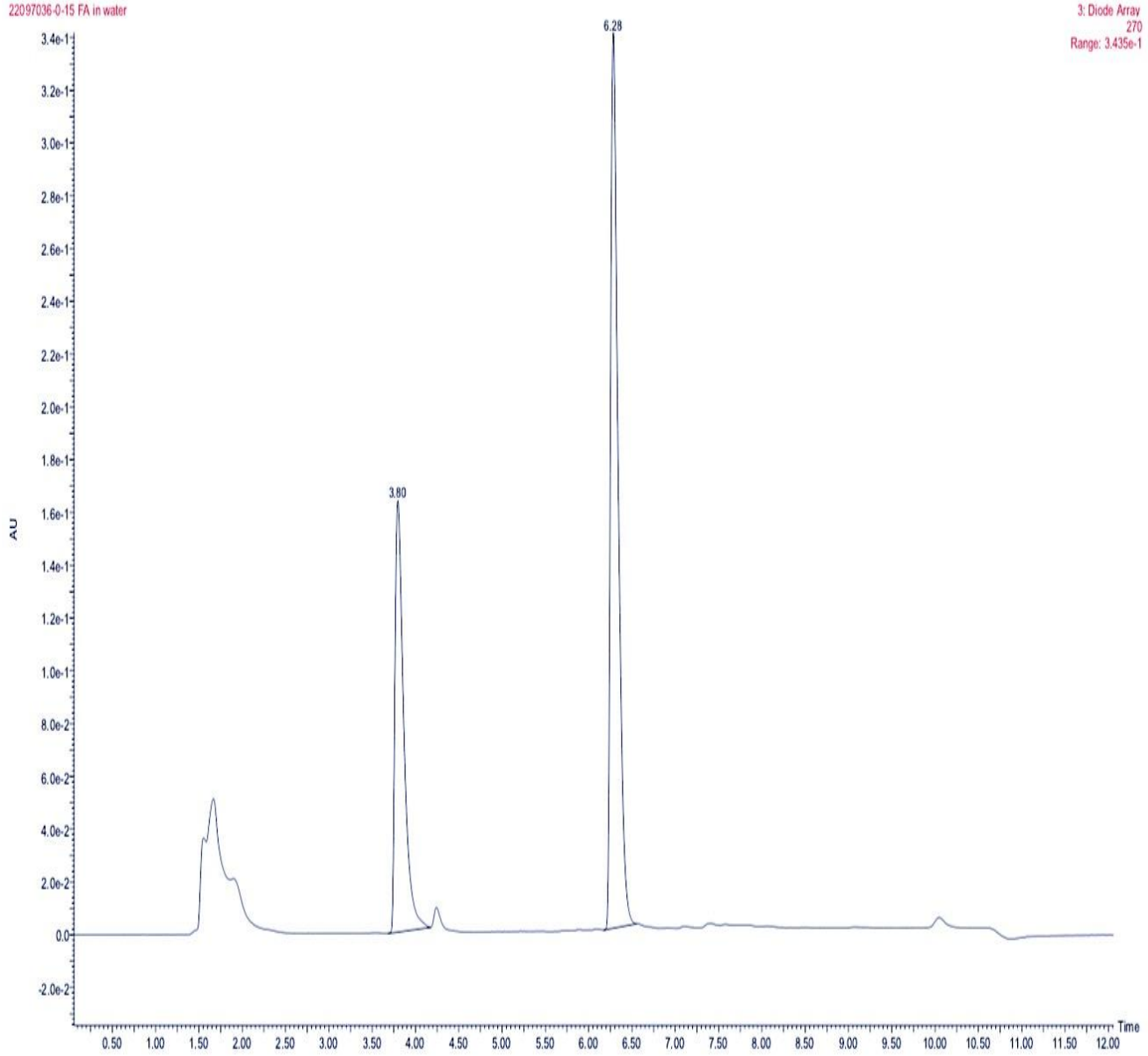


Figure 11: Robustness for Mobile phase- A: 0.15%Formic acid in Water

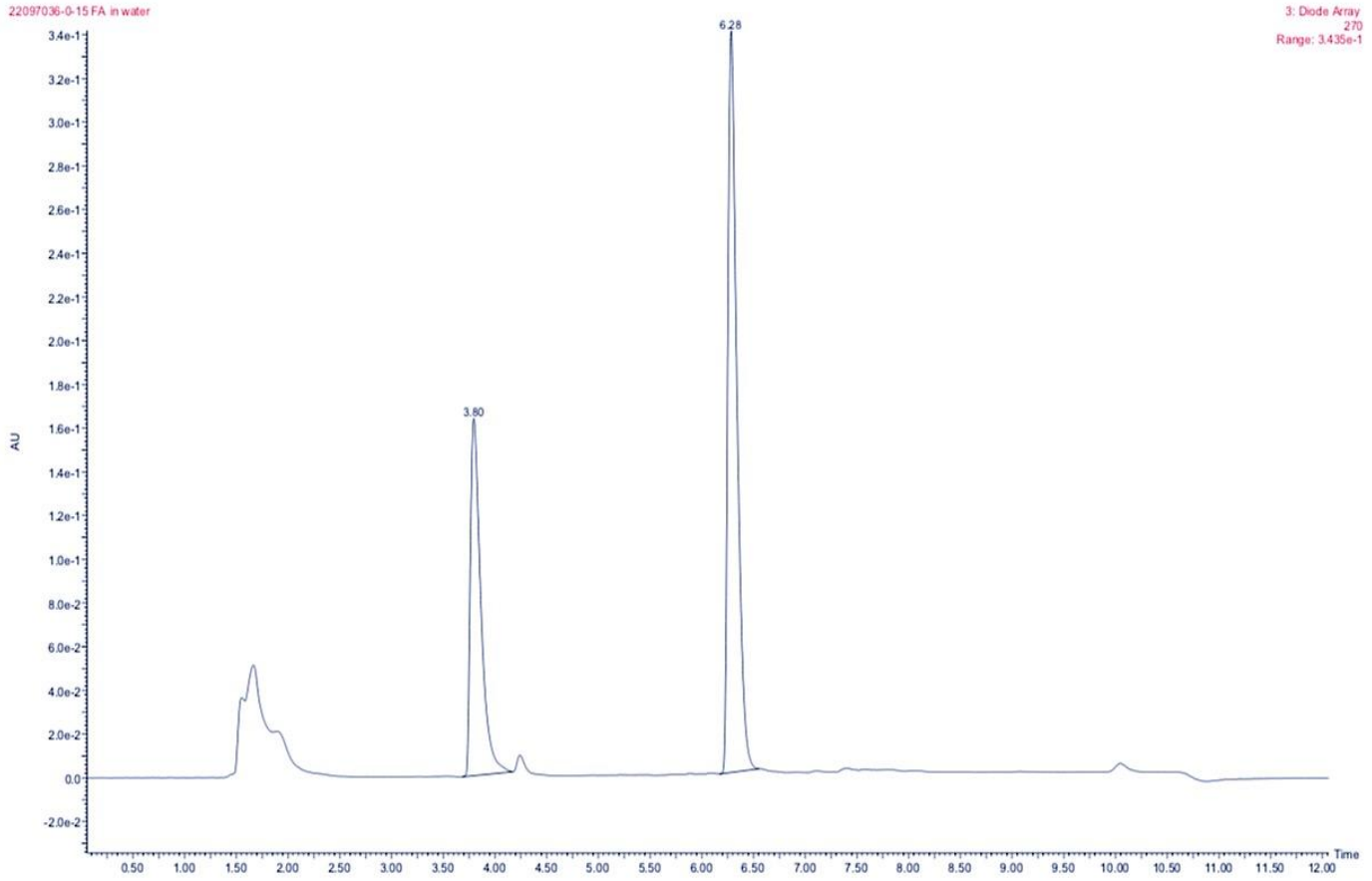


Figure 12: Injection Reproducibility - 3µl

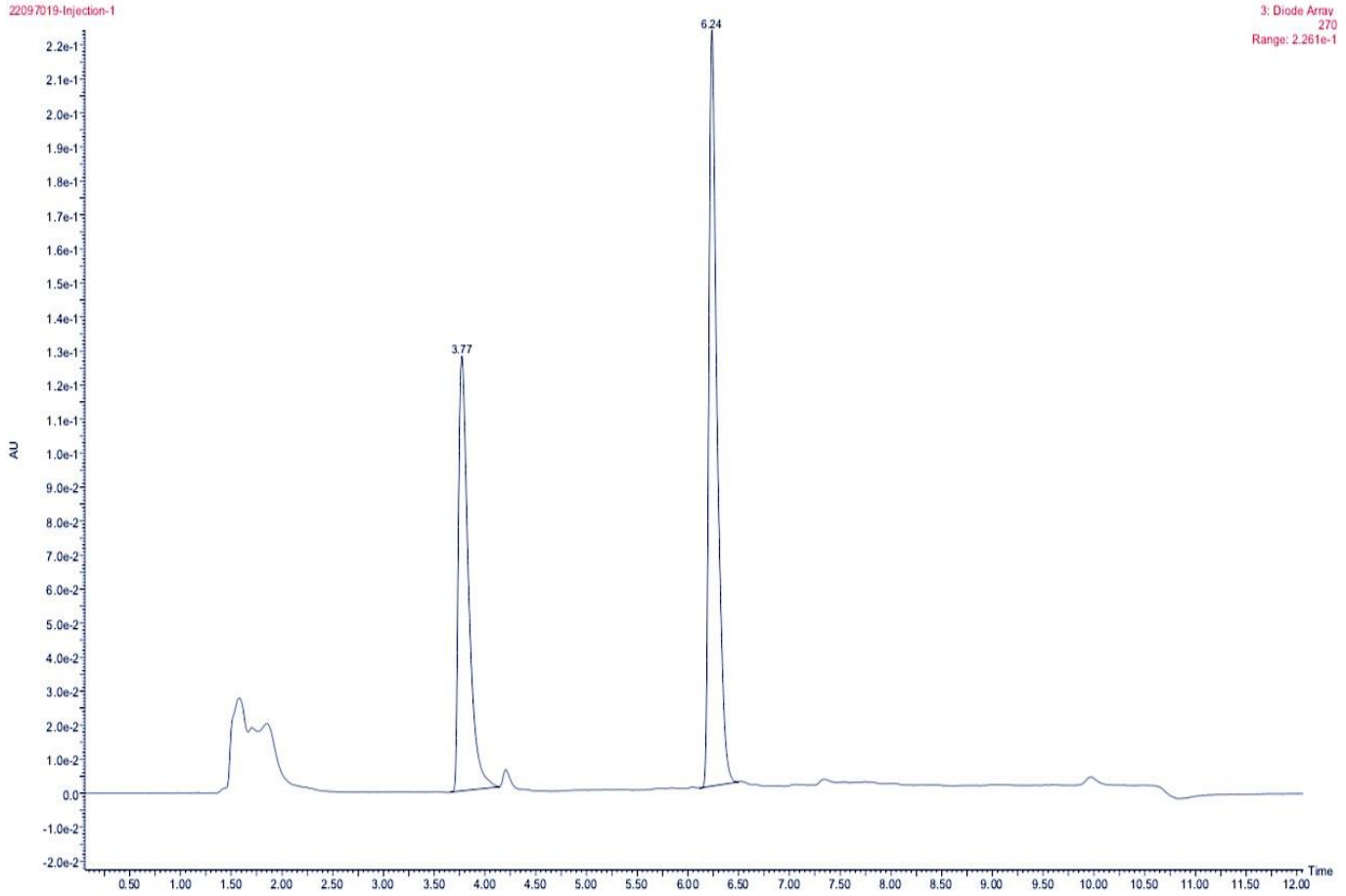
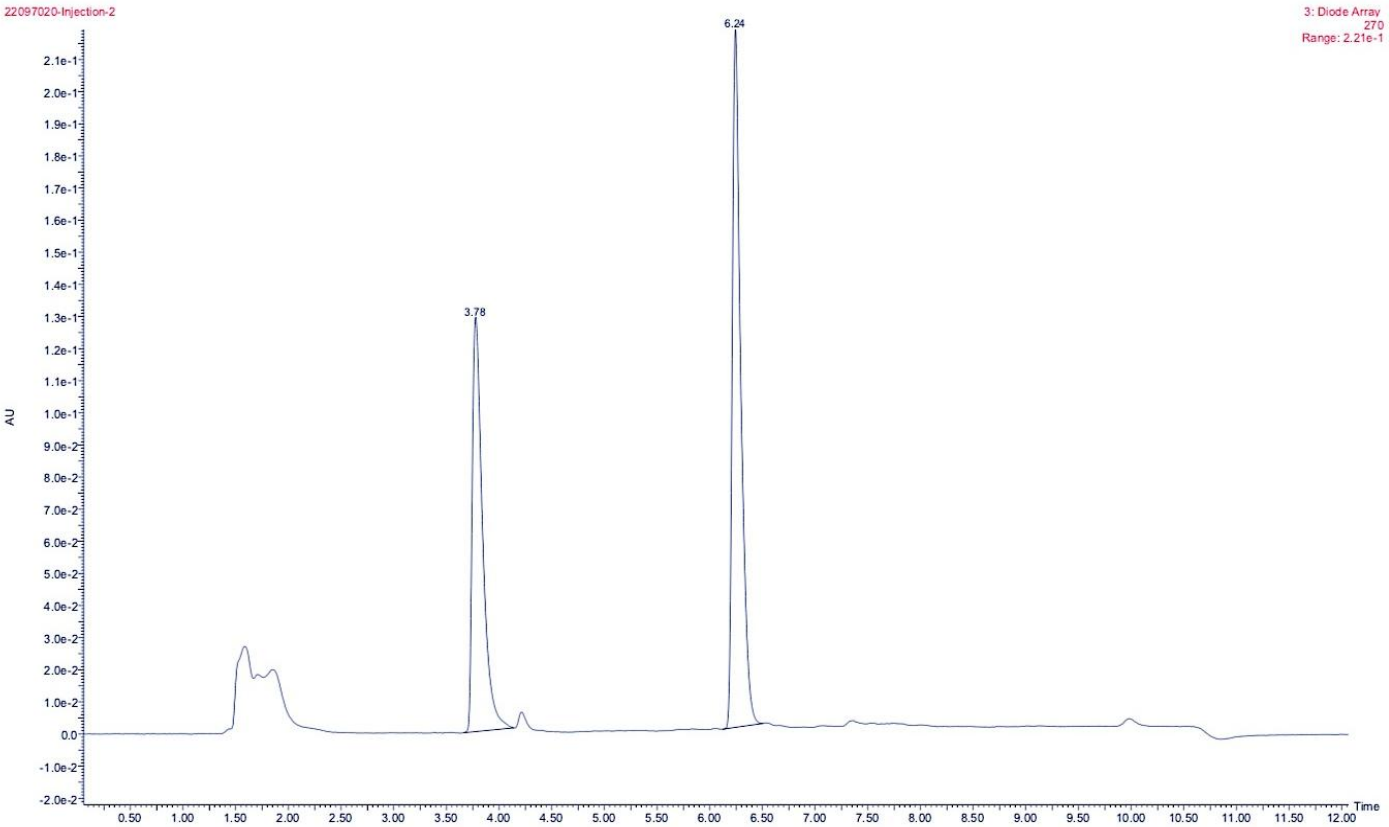


Figure 13: Injection Reproducibility - 6µl



CONCLUSION

A rapid, precise, accurate, and economical UPLC method was established for concurrent quantification of olanzapine and carbamazepine in accordance with USFDA. In addition to providing a short run time of 12 minutes, the retention times of olanzapine and carbamazepine were observed at 3.75 and 6.17 minutes, respectively and the flow rate was (0.4 mLmin $^{-1}$). These attributes contribute to the method's effectiveness, optimizing both time and cost of analysis. This validated method offers sensitivity, selectivity, reproducibility, and rapid analysis olanzapine and carbamazepine in active pharmaceutical ingredient (API). The method demonstrated accuracy and precision within permissible limits and hence, the validated bioanalytical method is reliable, precise, and accurate, making it suitable for routine application.

In routine practice, it is essential to establish methods that can analyze multiple samples with shorter runtime, while maintaining good accuracy and precision. Chromatographic technique facilitates the production of large amounts of quality data, thereby providing a versatile and valuable tool. In the present work UPLC methods were optimized for simultaneous estimation of olanzapine and carbamazepine by using bio analytical method. Literature survey revealed that no HPLC method was available to quantify the olanzapine and carbamazepine.

The developed method was validated using various parameters. The findings indicate that the proposed UPLC method was found to be simple, precise, economical, and suitable for determination. Hence, it can be routinely adopted for quantification of olanzapine and carbamazepine in the formulation.

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Conflict of interest

The authors hereby declare that there are no known financial or non-financial conflicts of interest that could have appeared to influence the results, interpretation, or presentation of this research work.

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