Postmortem Detection of Olanzapine and Clozapine Residues in Rat Brain following different Modes of Death using HPLC-UV Technique

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Abstract

The aim of this study was directed to develop and validate an HPLC with UV detection method for quantification of Olanzapine (OLZ) and Clozapine (CLZ) in rat brain tissue homogenate following different modes of death, for this purpose sixty adult male albino rats were classified into four equal groups; first group (control) received distilled water, second group received OLZ at a dose of 5 mg/kg B.wt, third group received CLZ at a dose of 20 mg/kg B.wt. and fourth group received both OLZ and CLZ at the same previously mentioned doses. The administration was carried out daily by gavage for four months. At the end of the experiment; the four main groups were classified according to mode of death into three equal subgroups; drowning group, electrocution group and starvation group. The analytes and risperidone (IS) were isolated from rat brain tissue homogenate by solid phase extraction on Waters Oasis HLB cartridges. The compound was separated on a kinetex C18 column (100×4.6 mm, 2.6 µm) using a mixture of acetonitrile (A), methanol (B) and 34 mM phosphate buffer pH 2.4 (C) as mobile phase at a flow rate of 1.0 mL/min while detection wavelength was 254 nm. Under these conditions, OLZ and CLZ were well separated and showed good linearities in the ranges of 0.5–100 ng/g for OLZ and 0.5–200 ng/g for CLZ with r ≥ 0.999. The intra-day and the inter-day precisions were better than 4.3 %. Recoveries of OLZ and CLZ were 90.1–94.1% and 86.5–90.5%, respectively. The residual detection study in the three examined groups showed that the sequence of OLZ or CLZ concentration from high to low was drowning > electrocution > starvation. Histopathological findings of brain tissue varied from slight to more sever degrees of lesions as depicted in co-administered group.

Keywords: Olanzapine, Clozapine, HPLC, Solid phase extraction, Mode of death.

Introduction

Antipsychotic drugs are of significant interest in the field of forensic toxicology due to their abuse potential and their involvement in poisoning and suicides. In recent years, many antipsychotic drugs became available in the market for their pharmaceutical uses, so it became necessary assays for analysis need to be investigated[1].

Antipsychotic medications have many uses, including psychological treatments for it is one of the most important foundations of schizophrenia and other psychoses treatments[2,3]. In addition, some phenothiazine derivatives such as acepromazine have many uses for many animals, such as cats, dogs and utilized extensively in horses as a pre-anaesthetic sedative[4].

Antipsychotics are usually categorized into two drug sections the first section of which is known antipsychotic first-generation (typical) and the second section known antipsychotic of the second generation (atypical)[5]. Antipsychotic first-generation (conventional antipsychotics) are first developed in the 1950 such as phenothiazines, butyrophenones (haloperidol),

droperidol and bromperidol[6]. They often indicated to as typical antipsychotic drugs due to their typically produce extrapyramidal effects as a direct or indirect result of blockade of D2 receptors[7]. Antipsychotic of the second generation are often indicated to as atypical antipsychotic drugs such as Clozapine, sulpiride, aripiprazole, Olanzapine and risperidone[7]. Olanzapine and Clozapine are similar in structure and are considered of atypical antipsychotics, which have many advantages, including their superior side-effect profiles with regard to extrapyramidal side effects[8,9]. The main metabolites for both of Olanzapine and Clozapine are N-desmethylolanzapine and N-desmethylclozapine while only the latter is pharmacologically active[9].

Numerous analytical methods are studies in the literature for quantification of Olanzapine in biological matrices including gas chromatography (GC) connected with (NPD) detection[10-12]. However, in most previous studies (HPLC) has been utilized with ultraviolet detection[13–23], coulometric detection[24], fluorescence detection[25] or electrochemical detection[26-29] for the quantification of Olanzapine and Clozapine in brain tissues or biological fluids. Recently, (LC–MS) were reported for the determination of the antipsychotic drugs in biological fluids[30–32]

and some LC-MS/MS studies were reported for the quantification of Olanzapine and Clozapine in biological fluids have been published[33–41]. This is the first study which have developed and validated a rapid and specific HPLC with UV detection method, utilizing solid phase extraction for quantitation of Olanzapine and Clozapine in rat brain over different modes of death with individual and oral co-administration besides the histopathological examination as an expected outcome.

Materials and Methods

Chemicals and reagents: All standards, chemicals and reagents had a purity of at least 99.99%, as certified by the manufacturer and all solutions were prepared fresh daily. All solvents were high performance liquid chromatography grade. The drugs under investigation are: Olanzapine (OLZ), Clozapine (CLZ) and risperidone (RIS) (internal standard, I.S.) that kindly supplied from Delta Pharma, 10th of Ramadan City, Egypt. Hydrochloric acid, sodium hydroxide was supplied from Sigma–Aldrich. Acetonitrile, methanol and orhophosphoric acid were of HPLC grade obtained from Merck. All other chemicals used were of analytical grade or higher.

Experimental animals, diets and housing conditions: Sixty adult male albino rats were separated into four equal groups (fifteen rats each) gained from laboratory animal breeding unit (Faculty of Veterinary Medicine, Zagazig University) weighting between 180 to 200 gm, animals were classified and dosed orally as depicated in (Table-1). The rats were stayed singly in a room temperature (25ºC), preserved on a 12-h dark/night cycle with easy access to food. The animals were hosted in laboratory conditions for fifteen days before the experiment going on.

Table-1
Classification of animals on the basis of weight and their oral doses

Groups	No. of rats	Dose		Duration (day)
		CLZ (20.0 mg/kg B.wt)	OLZ (5.0 mg/kg B.wt)	
1st group	15	-	-	120
2nd group	15	-	+ve	120
3rd group	15	+ve	-	120
4th group	15	+ve	+ve	120

Experimental designs: After we got to the end of the experiment, (after 120 days); each of the four main groups were classified according to mode of death into three equal subgroups; drowning, electrocution and starvation groups (five rats each) as following: i. The first subgroup (drowning group); where prepared container filled with tap water and then the rat were put out with a push down until death. ii. The second subgroup (electrocution group); two hundred and twenty volts (220V) was applied to the tail and left anterior extremity. iii. The third subgroup (starvation group); the rats housed individually in metal-wire bottom cages with deprivation of food and water until death.

Brain Tissue Sampling: Brain tissues of the different groups were split into two specimens.

Residual detection: The first specimen is processed in a safe way to prevent any contact with any external surface and then subsequently kept at -20ºC till transported to the laboratory for analysis using HPLC.

Histopathological examination: The second specimen of each brain was immersed in 10% buffer formalin and after that used for histopathological investigation.

HPLC conditions: Agilent 1100 system with G1314A-90004 UV detector was utilized for analysis, and the detection wavelength was 254 nm, column oven temperature 25ºC, and an online solvent degasser system was used. A kinetex C 18 (100×4.6 mm, 2.6 µm) column, the mobile phase was composed of acetonitrile (A), methanol (B) and 34 mM phosphate buffer pH 2.4(C) in gradient mode (0 ~ 4 min, A: 10%, B: 10%; C 80%, 4~ 12 min, A: 70% , B: 10% , C: 20%,) While during the experiment the flow rate was 1 mL/min.

Preparation of stock and working dilution and spiking solutions: All standard stock solutions were prepared for each of Olanzapine, Clozapine and risperidone (internal standard) at the desired concentrations (1.0 mg/ml) by dissolving a specific amount of this compounds in methanol Then diluting these solutions with methanol: water (60:40, v/v) to get a different sequences from working solutions for each of OLZ, CLZ and RIS (internal standard) in the required concentration range (100 µg/ml). All prepared solutions were kept at 2–8ºC but if used were transported to room temperature. The Calibration standards (CS) composed of seven different concentrations (0.5, 1, 5, 10, 20, 50 and 100 ng/g for OLZ; 0.5, 2.5, 5, 20, 50, 100 and 200 ng/g for CLZ) and quality control (QC) samples composed of three different levels low, medium and high (0.60 , 6.0 and 60.0 ng/g for OLZ; 0.60, 60.0 and 150.0 ng/g for CLZ) were prepared by spiking the blank brain tissue samples with respective working solutions. All of the brain tissue samples were kept at -20ºC even analysis time.

Sample preparation or Extraction procedure: Solid-phase procedure for extract each of OLZ or CLZ from all brain tissue samples was developed by using cartridges which activated and conditioned with 2.0 m L of methanol, then added 2.0 m L of purified water. The brain tissue samples for all rat that died in different ways were cut into small pieces and after that homogenized in a size of purified water (in ml) equal to twice the weight (in g) of the tissue. The 500 µl brain homogenate sample (analyte standard solution for blank brain homogenate sample), 50 µl of risperidone (IS) and 500 µl 0.1N HCL were

added then vortexed well for 3 min thereafter centrifuged for 15 min at 9000 rpm. Finally the supernatant of the mixture obtained after centrifugation was loaded onto the conditional cartridge, thereafter, washing by 1.0 ml of purified water two times then added 1.0 ml of 5% methanol in water, finally sucked dry and eluted with 1.0 ml of methanol. The eluent was dried under vacuum for 5 min, dissolved again with 250 µl of methanol and then 2µl was injected into the HPLC.

Method Validation: Specificity: The specificity of this method was estimated by assay control brain homogenate samples, control brain homogenate samples spiked with OLZ, CLZ at LLOQ concentration and IS and rat brain tissue samples after oral administration of analytes.

Calibration Curves and Lower Limit of Quantification: It was established calibration curves using seven concentrations of each of Olanzapine and Clozapine in brain samples by plotting the ratio of the peak area of OLZ or CLZ to that of the risperidone (internal standard) against the concentrations of OLZ or CLZ and then it was evaluated through linear regression analysis. The lower limit of quantification expressed by the symbol (LLOQ) was known as the lowest concentration of analyte in a sample which provided a peak area with a signal-to-noise ratio higher than 10.

Precision and Accuracy: Intra-day precision which expressed as the relative standard deviation (RSD %) and accuracy which expressed as the relative error (RE %) for this analytical method were estimated by measuring the concentrations of QC samples of tissue homogenates (0.60, 6.0 and 60.00 ng/g for Olanzapine; 0.60, 60.0 and 150.0 ng/g for Clozapine) five times on the same day while the same steps were performed one time daily for five sequential days to determine inter-day accuracy and precision.

Extraction Recovery: For estimation of the extraction recovery of OLZ and CLZ at three QC samples of tissue homogenates which prepared as described earlier this was done by comparing the concentrations of both OLZ and CLZ after extraction procedure with concentrations of non-extracted standards.

Stability: To examine the stability of OLZ or CLZ in brain samples were performed with concentrations of (6 and 60 ng⁄g OLZ; 60 and 150 ng⁄g CLZ) in a several different conditions, including three freeze-thaw cycles. At each cycle, the samples are transferred from the freezer to room temperature and left for a sufficient period of time to melt and re-freeze them again at -20 ℃. while long term stability was estimated after keeping the samples of (6 and 60 ng⁄g OLZ; 60 and 150 ng⁄g CLZ) at -20 ℃ for thirty days.

Histopathological Examination: After all rats were died by different ways of death as described above, the second specimens of each brain were immersed directly in a solution of 10% diluted formalin then embedded in paraffin wax for histopathological examination[42].

Statistics: The obtained data were analyzed and graphically represented by two way analysis of variance (ANOVA). The data were analyzed using T- test to determine the statistical significance of difference among groups.

Results and Discussion

Specificity and chromatography: The optimum chromatographic conditions (mobile phase composition, time required for flow rate, type of analytical column and the volume injected) were established by varying one variable and observing its effect on the sensitivity, separation, run time and good peak shapes for the OLZ, CLZ and RIS (IS). Chromatograms for determination of OLZ and CLZ in brain tissue are shown in (Figure-1 and 2). The retention time of OLZ, CLZ, and IS were 1.78, 8.28 and 7.66 min, respectively.

Linearity of Calibration Curve and Lower Limit of Quantification: Good linearity was achieved over the concentrations in the range of 0.5 to 100 ng⁄g for Olanzapine and 0.5 to 200 ng⁄g for Clozapine, and coefficient of correlation were found to be better than 0.999. The data of linearity are listed in (Table-2). And the value of LLOQ for each of Olanzapine and Clozapine equal to be 0.5 ng⁄g

Table-2
Validation data of the proposed method for quantification of OLZ and CLZ in brain tissue

Parameters	Brain	
	OLZ	CLZ
Linear range (ng/g)	0.5-100	0.5-200
Slope	0.0292	0.0226
Slope standard errors	0.0001	0.0002
Intercept	0.0199	0.0040
Intercept standard errors	0.0055	0.0020
Correlation coefficient	0.9998	0.9997
Number of data points	7	7
LLOQ(ng/g)	0.5	0.5

Precision and Accuracy: The results of intra or inter-day precision and accuracy for all QC in brain tissue homogenates samples were tabulated in (Table-3). Through these results it proved that the current method has acceptable precision and accuracy for all brain tissue homogenates samples so the results of the intra or inter-day accuracy at three levels were among -2~0.666% and -4.670~.0616% for Olanzapine and Clozapine respectively while the relative standard deviation of intra-day was less than 3.5 % and 3.2%, and the relative standard deviation of inter-day precision was less than 4.1 % and 4.3% for Olanzapine and Clozapine respectively.

Extraction Recovery: Data of extraction recovery for OLZ and CLZ at three QC concentrations were tabulated in (Table-4). This showed that the extraction recoveries were more than 86% for two analytes and these results consider acceptable.

Stability: The samples of moderate QC concentrations (6 and 60 ng⁄g OLZ and 60 and 150 ng⁄g CLZ) in rat brain homogenate exhibited good stability under the diverse storage conditions mentioned above (long term storage and freeze-thaw); all samples were stable in case stored at -20 ºC for a period was 30 days. There was no considerable difference can be determined given that the bias in concentration was within ± 15 % of the acceptable values.

Residual detection: The HPLC method mentioned above has been utilized to set the concentrations of OLZ and CLZ in rat brain homogenate after the rats were given fixed doses from the two drugs over the long term of the experiment then scheduling all data about concentration of two drugs in the samples at different mode of death (drowning, starvation and electrocution) in (Table-5). The representative of OLZ and CLZ graphs creating from the analysis all samples after the long-term treatment of two analyte is illustrated in Figures-3-11, which revealed that the sequence of the two drug concentration in various modes of death groups from high to low was drowning group followed by electrocution group and then starvation group. These observations indicated that OLZ and CLZ concentration in brain tissue affected by different mode of death. On nearly similar ground those findings with previous studies[1,43]. Also According to our experimental study we recorded that in all groups, the concentrations of OLZ were higher than CLZ in brain tissues and these results came in harmony with the reported literatures values[40,44]. In addition the concentrations of OLZ and CLZ in all groups were significantly altered by nature of oral administration remarkably decreased in case of co-administration, which suggested that the absorption of OLZ or CLZ in rat body became weaker in the presence of both drugs and this is compatible to some studies, which used both Clozapine-risperidone and the results indicate an increase in the concentration plasmatic concentration of Clozapine when co-administrated with risperidone[45] and there are some reports indicate to increase plasmatic level of Clozapine and risperidone in the presence of fluoxetine[46].

Histopathological findings: Brain section of control untreated rat in drowning group showed pyknotic nuclei of the necrotic neurons (arrowheads) with engorgement of the cerebral capillaries with erythrocytes (arrows) and slight vacuolation of the cerebral neuropil (Figure-12). On the other hand, microscopic investigation for brain section of drowning group treated with OLZ showed degenerated nerve cells (Figure-13), while the treatment with CLZ showed congested blood vessel in the white matter of the cerebral cortex and hemorrhage (Figure-14), as well as the treatment with combined of OLZ and CLZ showed ischemic shrunken neurons with marked pyknosis of its nuclei (Figure-15), while in case of electrocusion group the brain section of control untreated rat showed multiple focal hemorrhagic areas appear as extravastaed erythrocytes (arrowheads) with vacuolation of the cerebral neuropil (Figure-16), and the rats treated with OLZ showed congestion of cerebral blood vessels with marked perivascular hemorrhage (Figure-17). Moreover, brain of electrocusion group the rat treated with CLZ showed parenchymal hemorrhage and few necrotic neurons (Figure-18), as well as the treatment with two drugs showed congestion of cerebral blood vessels with marked perivascular hemorrhage (Figure-19). Additionally, histological investigation of brain section of control untreated rat in starvation group showed multiple pyknotic nuclei of the degenerated neurons (arrowheads) with marked vacuolation of the cerebral neuropil (Figure-20) and rats treated with OLZ showed degenerated neurons (Figure-21). But group treated by CLZ showed severe congestion in the meningeal blood vessels (Figure-22), and when treated with OLZ and CLZ showed few vacuolation of the neuropil with presence of extravastaed erythrocytes (Figure-23). The results of this study varied from slight to sever changes in brain tissue with the different modes of death but in the case of co-adminstration of the drugs, the effect was more obvious than in the case of a single adminstration. However, previuos study have shown that the use of antipsychotic drugs for long periods causing cortical thinning, which resulted in the gradual loss of brain tissue[47], also another study have shown that chronic use of both haloperidol and olanzapine caused a significant decrease in the size of the entire brain[48] while on the contrary the use of risperidone does not affect the histological architecture of the brain[49]. Meanwhile it was found that the use of haloperidol causing major damage in the cerebral cortex, internal capsule, and substantia nigra[50].

Table-3
Intra- and inter precision and accuracy for Olanzapine and Clozapine (n=5)

Matrix	Drug Name	Conc. added (ng/g)	Intra - Day			Inter - Day		
			Mean conc. found (ng/g)	R.S.D (%)	Error (%)	Mean conc. found (ng/g)	R.S.D (%)	Error (%)
Brain	OLZ	0.6	0.602	3.404	0.333	0.604	4.030	0.666
		6	5.940	2.553	-1	5.880	2.522	-2
		60	60.310	2.154	0.516	60.370	1.766	0.616
	CLZ	0.6	0.571	3.127	-4.670	0.588	4.234	-2
		60	57.970	2.280	-3.383	58.170	2.995	-3.050
		150	150.470	1.184	0.313	149.610	1.484	-0.260

Table-4
Extraction recoveries for Olanzapine and Clozapine (n=5)

Matrix	Drug Name	Conc. added (ng/g)	Mean conc. found (ng/g)	Recovery (%)	R.S.D (%)
Brain	OLZ	0.6	0.565	94.17	4.15
		6	5.515	91.91	2.86
		60	54.1	90.17	3.69
	CLZ	0.6	0.537	89.64	4.74
		60	54.34	90.57	2.91
		150	129.81	86.53	4.44

Table-5
Residual detection of Olanzapine and Clozapine following different modes of death

Drug	Drowning	Electrocution	Starvation
OLZ in oral single administration	877.96 ± 5.56^{Aa}	750.36 ± 2.43^{Ba}	788.91 ± 2.89^{Ca}
CLZ in oral single administration	135.21 ± 1.73^{Ac}	83.91 ± 1.7^{Bb}	61.81 ± 1.2^{Cb}
OLZ in oral co-administration	220.66 ± 2.33^{Ab}	83.31 ± 1.42^{Bb}	66.6 ± 1.84^{Cb}
CLZ in oral co-administration	49.56 ± 1.17^{Ad}	46.61 ± 1.13^{Ac}	34.09 ± 1.15^{Bc}

A>B>C concentration arrangement of olanzapine or clozapine in different group (Drowning, Electrocution or Starvation). a> b > c > d concentration arrangement of olanzapine or clozapine in the same group.
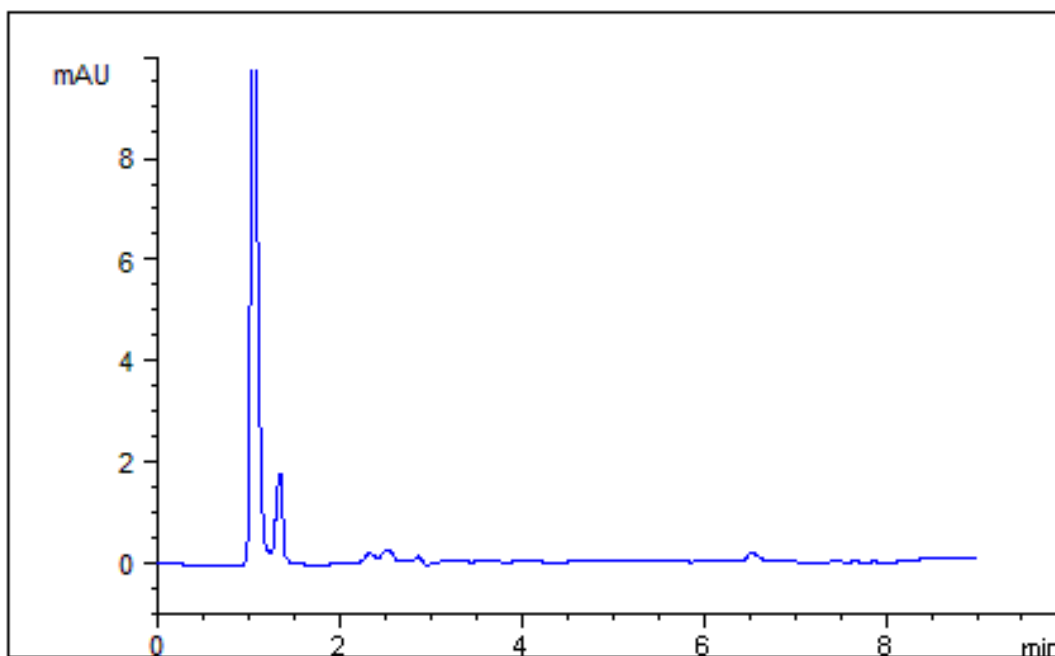
Figure-1
Illustrative chromatograms acquired from blank rat brain
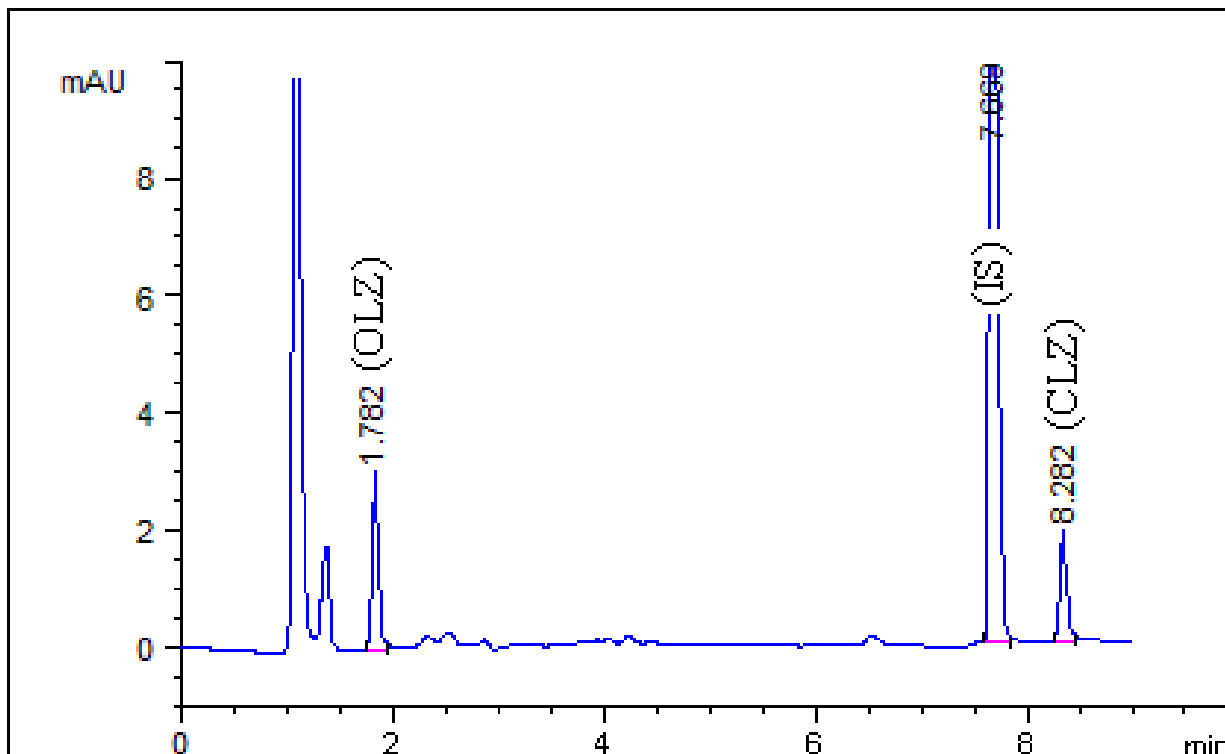
Figure-2
Illustrative chromatograms acquired from Brain homogenate spiked in accordance with (0.5 ng/g) concentration of two OLZ, CLZ and RIS (internal standard) (100.0 ng/ml)
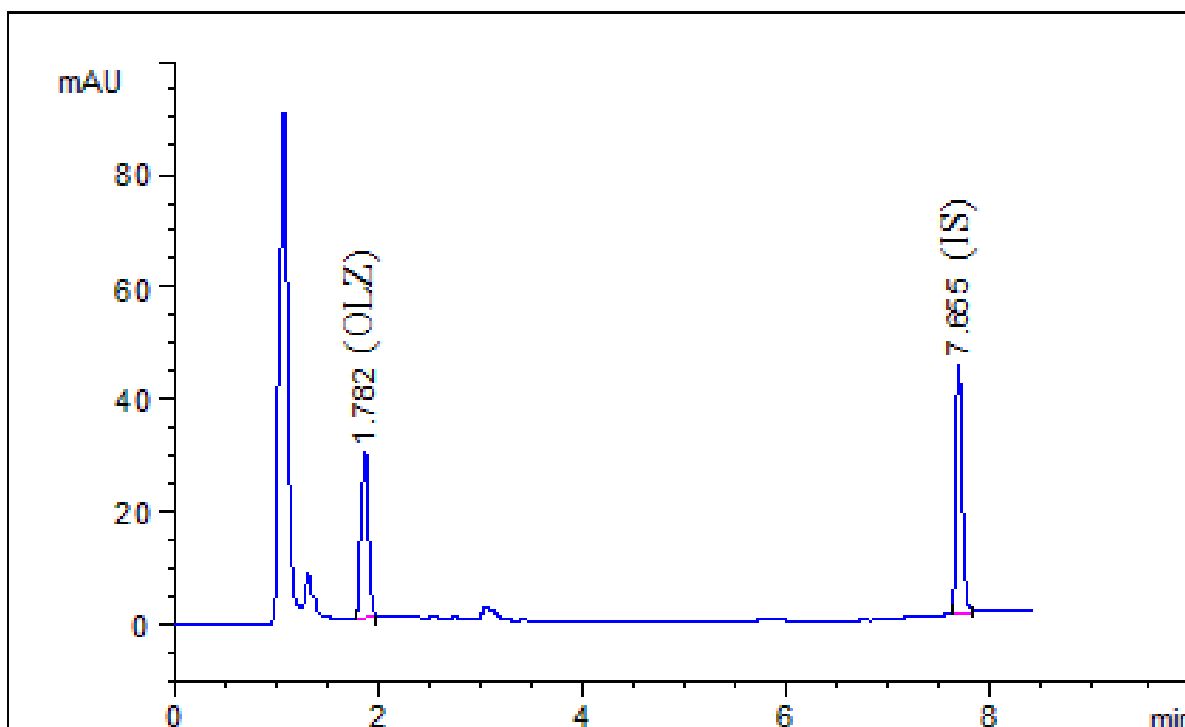
Figure-3
Illustrative chromatograms acquired from drowning group: A rat received a single dose of OLZ (5 mg/kg/day)
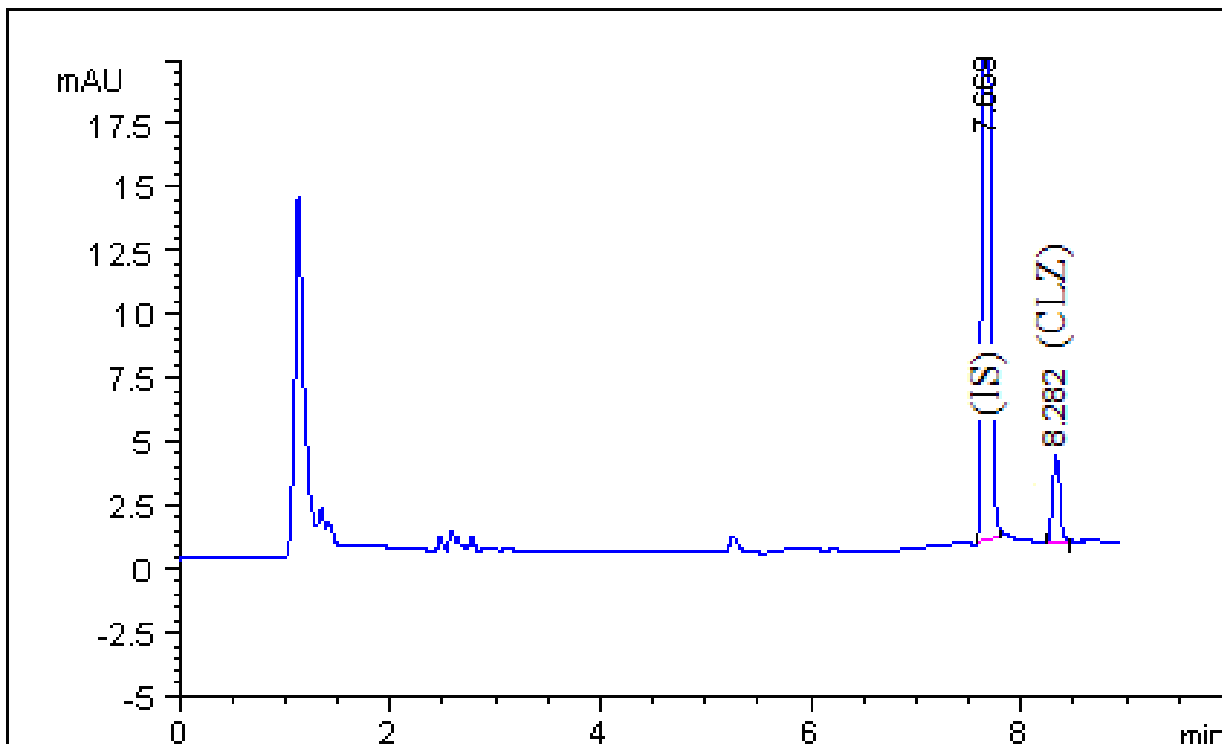
Figure-4
Illustrative chromatograms acquired from drowning group: A rat received a single dose of CLZ (20.0 mg/kg/day)
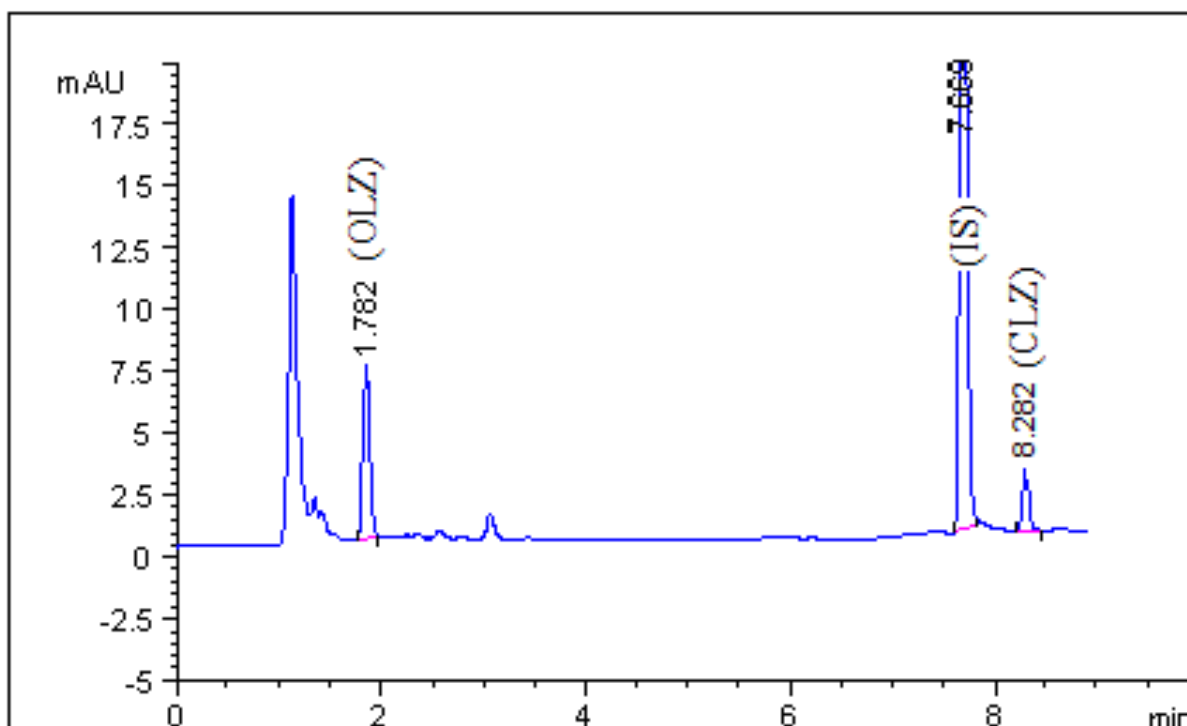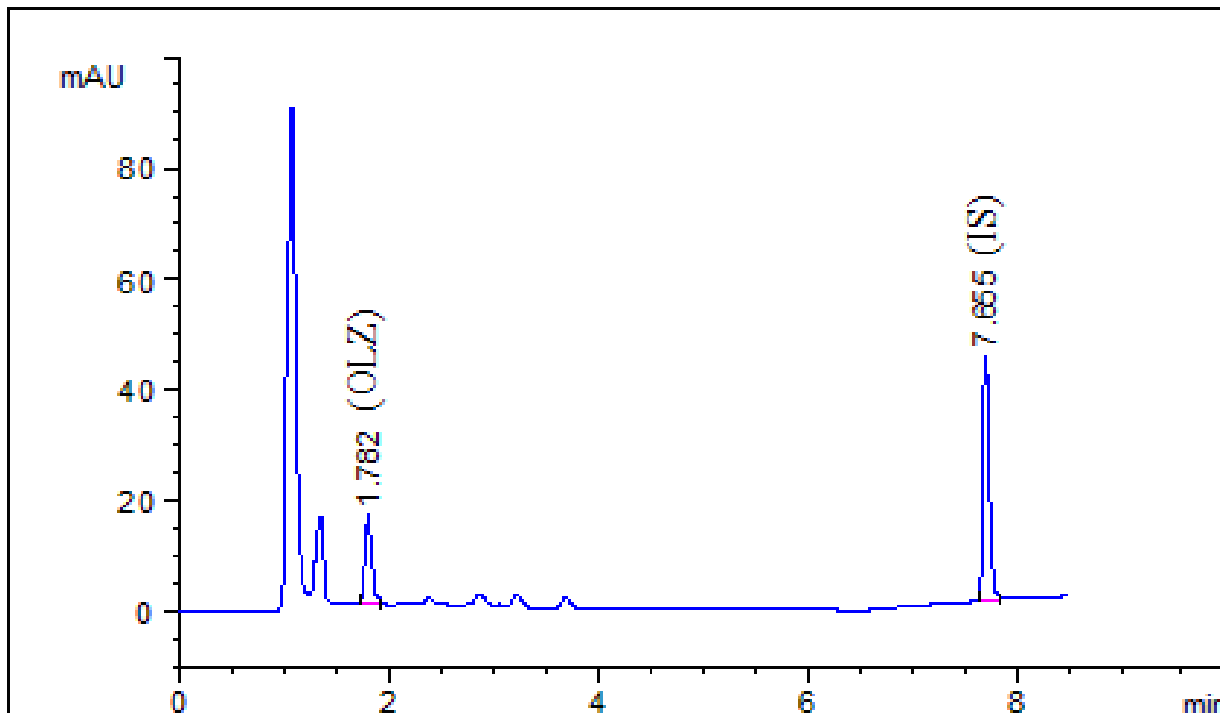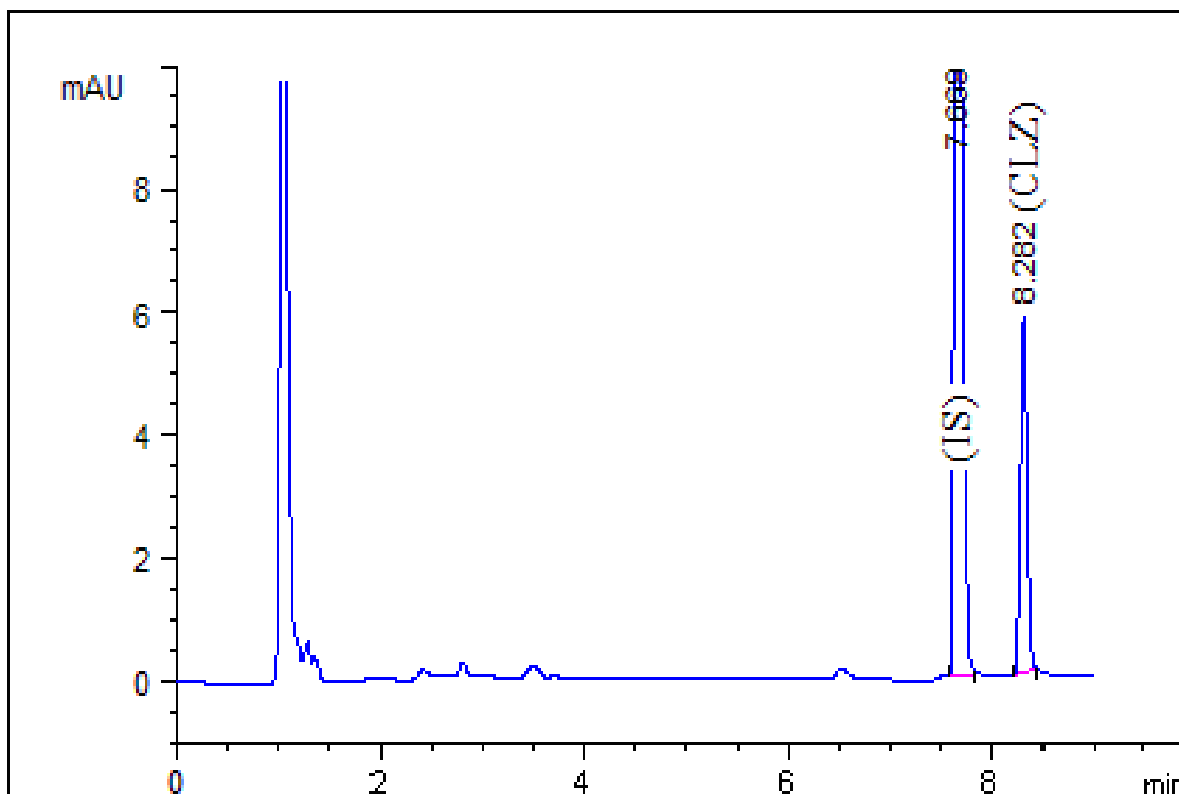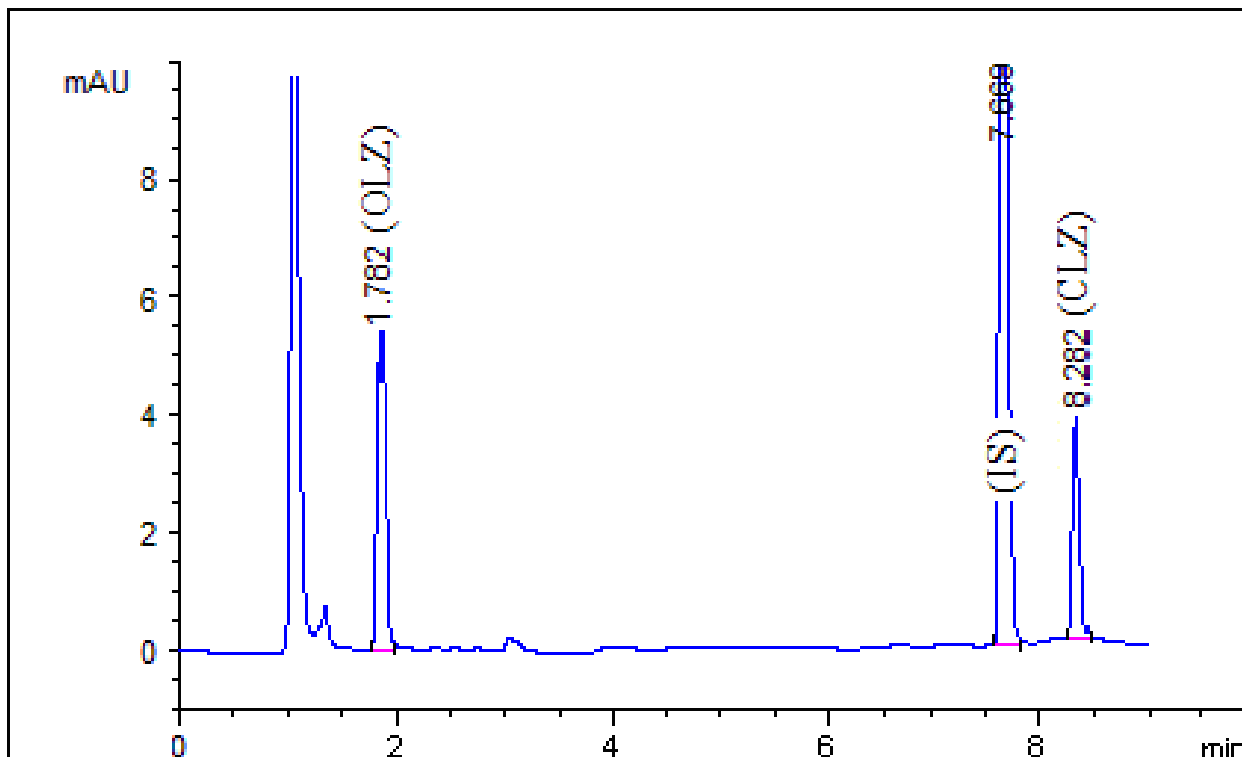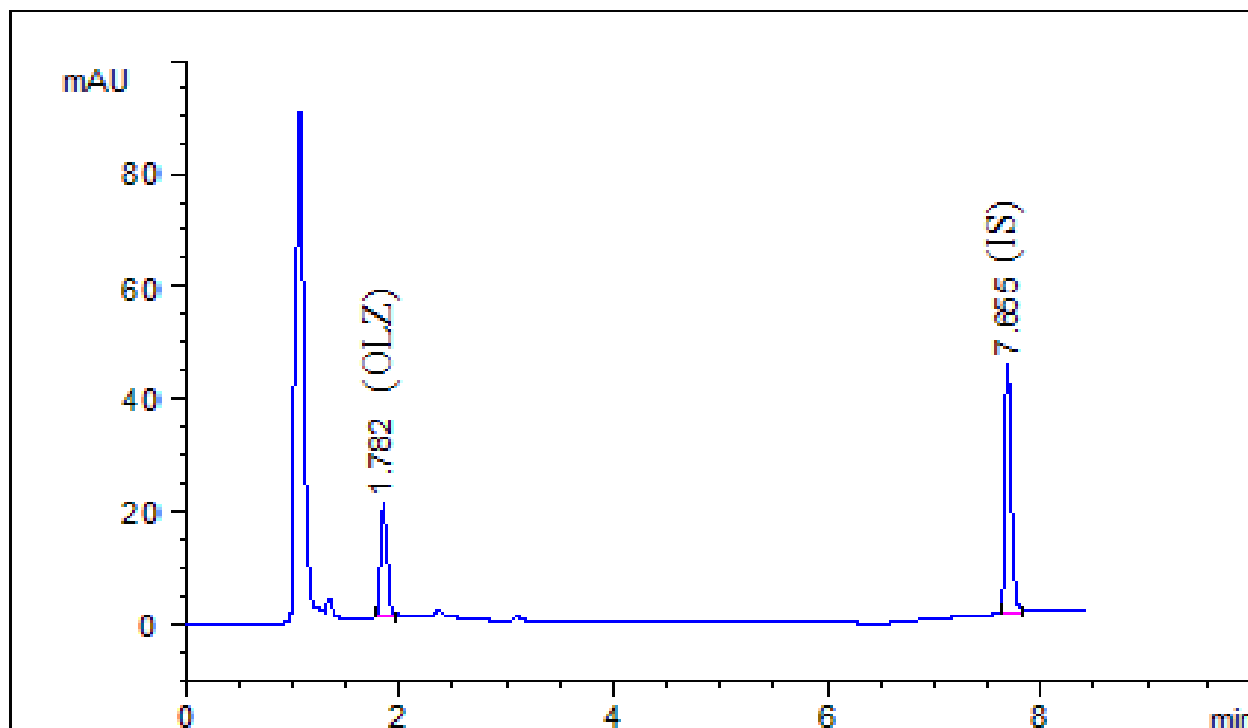

Figure- 5
Illustrative chromatograms acquired from drowning group: A rat received a dose both of OLZ (5 mg/kg/day) and CLZ (20.0 mg/kg/day)

Figure- 6
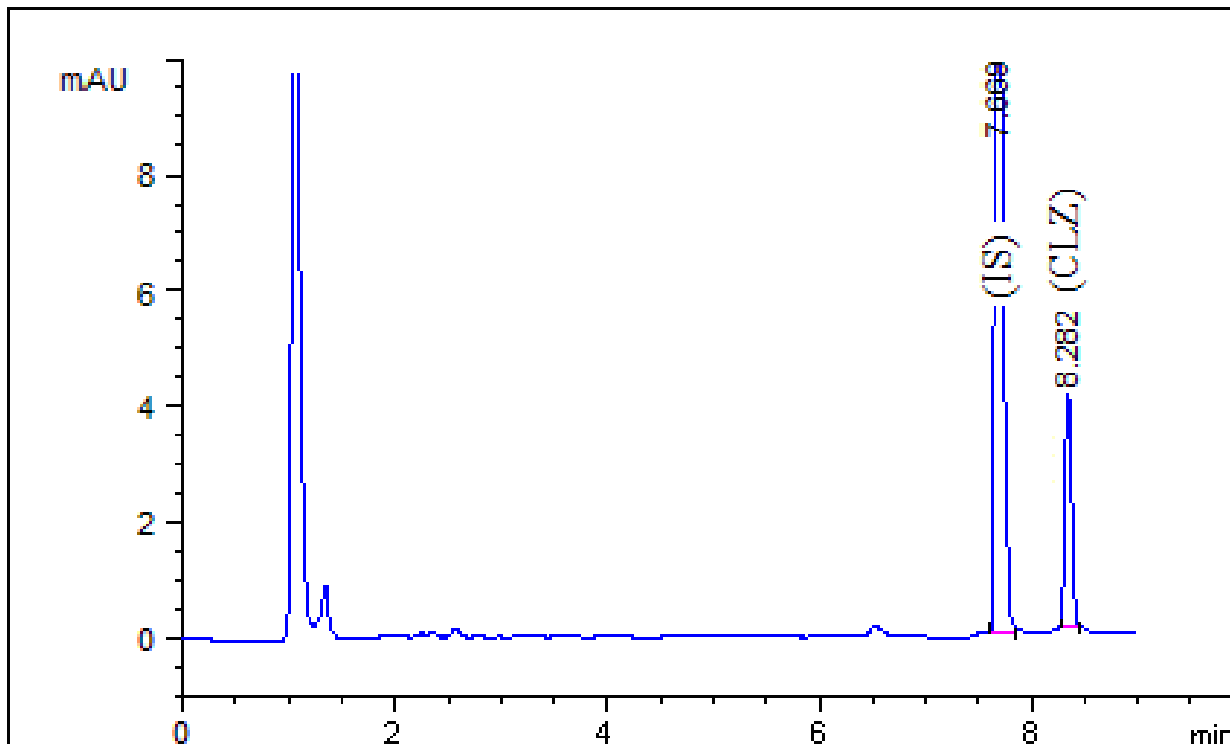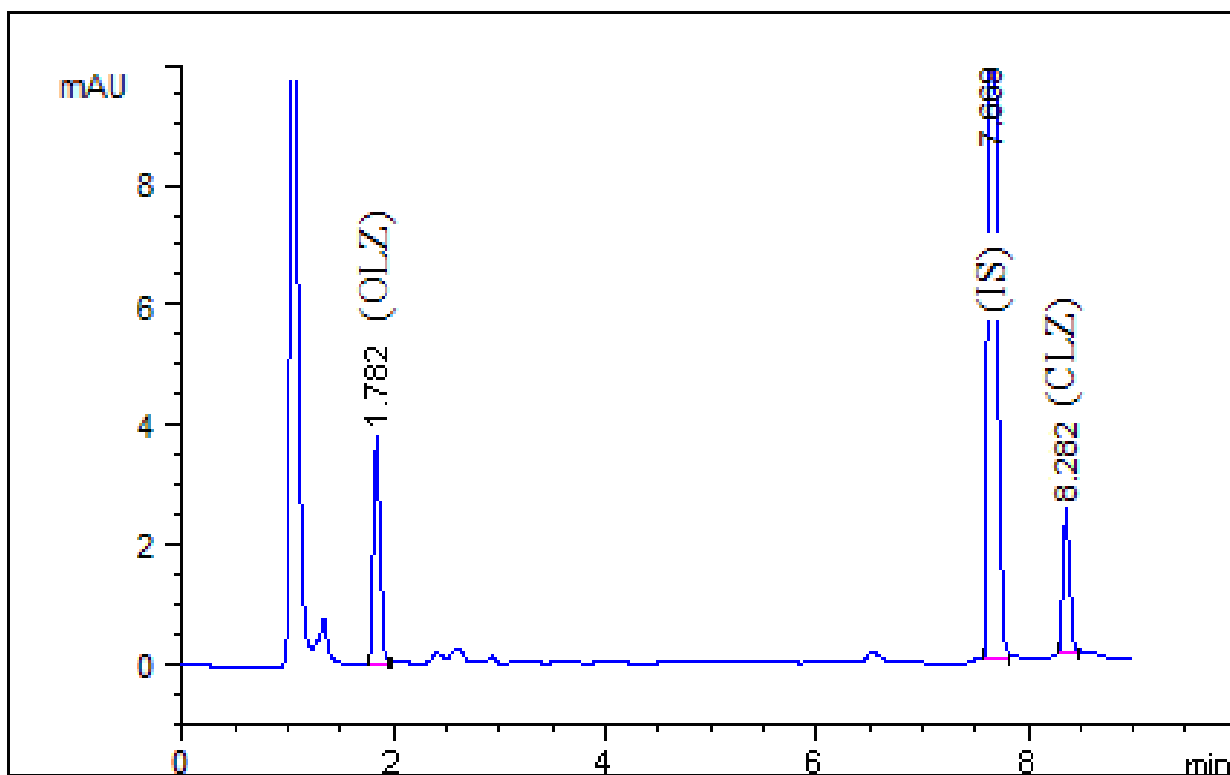**Illustrative chromatograms acquired from electrocution group: A rat received a single dose of OLZ (5 mg/kg/day)**

Figure-7
Illustrative chromatograms acquired from electrocution group: A rat received a single dose of CLZ (20.0 mg/kg/day)

Figure-8
Illustrative chromatograms acquired from electrocution group: A rat received a dose both of OLZ (5 mg/kg/day) and CLZ (20.0 mg/kg/day)
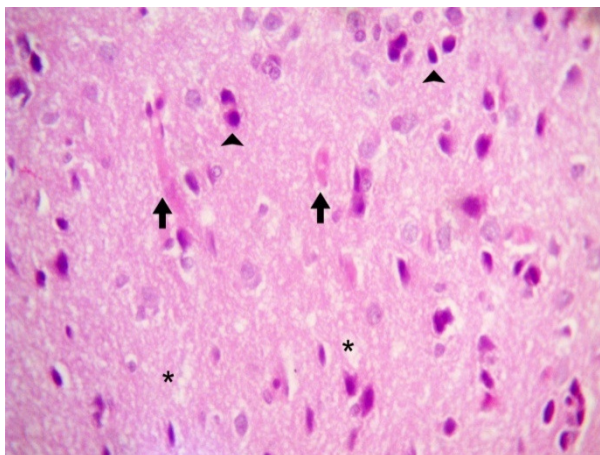

Figure-9
Illustrative chromatograms acquired from starvation group: A rat received a single dose of OLZ (5 mg/kg/day)

Figure-10
Illustrative chromatograms acquired from starvation group: A rat received a single dose of CLZ (20.0 mg/kg/day)

Figure-11
Illustrative chromatograms acquired from starvation group: A rat received a dose both of OLZ (5 mg/kg/day) and CLZ (20.0 mg/kg/day)

Figure-12
Brain of control, untreated rat, in drowning group showing pyknotic nuclei of the necrotic neurons (arrowheads) with engorgement of the cerebral capillaries with erythrocytes (arrows) and slight vacuolation of the cerebral neuropil (asterisks), H&E X400
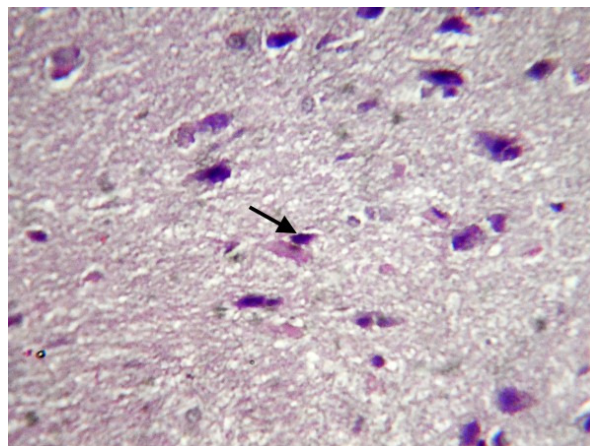

Figure-15
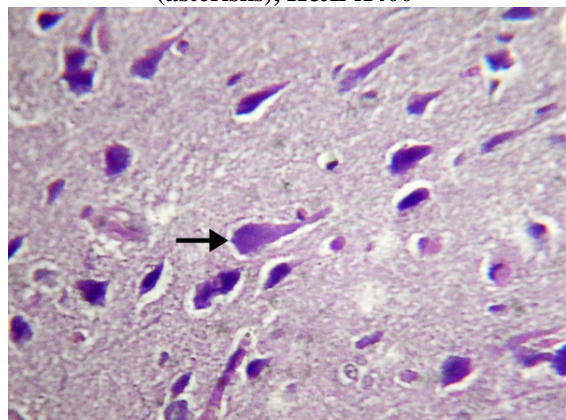**Brain of rat treated with, OLZ and CLZ in drowning group showing ischemic shrunken neuron with pyknotic nuclei (arrow), HE x400**

Figure-13
Brain of rat treated with, OLZ in drowning group showing degenerated nerve cell (arrow), HE x400
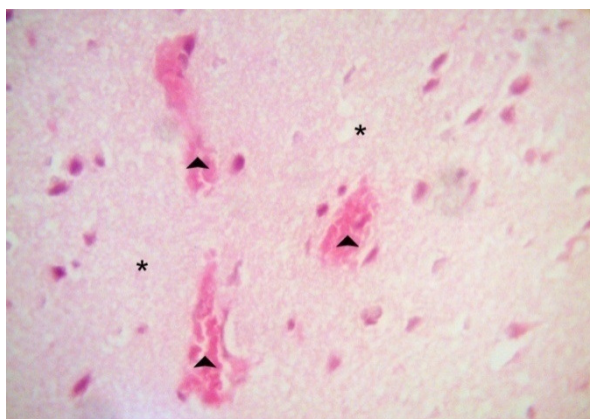

Figure-16
Brain of control, untreated rat, in electrocution group showing multiple focal hemorrhagic areas appear as extravastaed erythrocytes (arrowheads) with vacuolation of the cerebral neuropil (asterisks), H&E X400
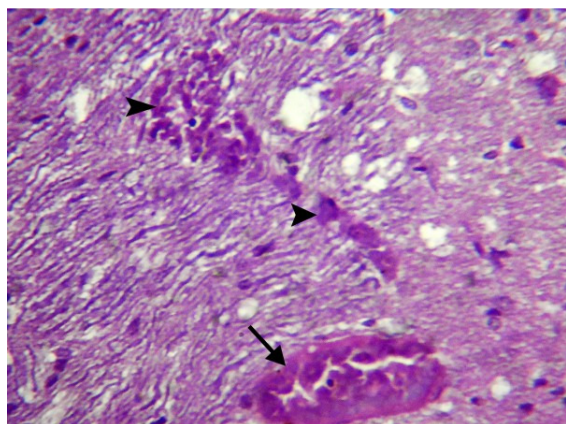

Figure-14
Brain of rat treated with, CLZ in drowning group showing congested blood vessel (arrow) and hemorrhage (arrowheads), HE x400
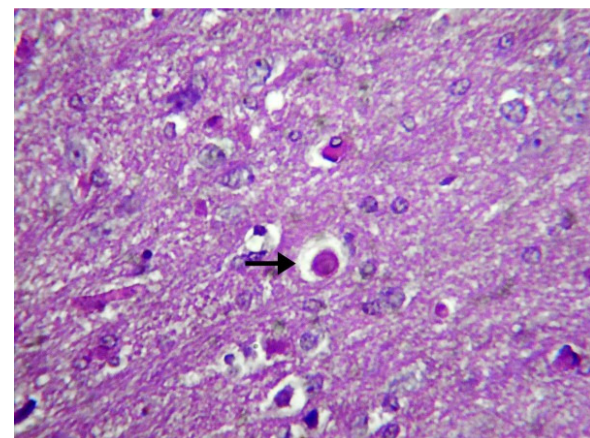

Figure-17
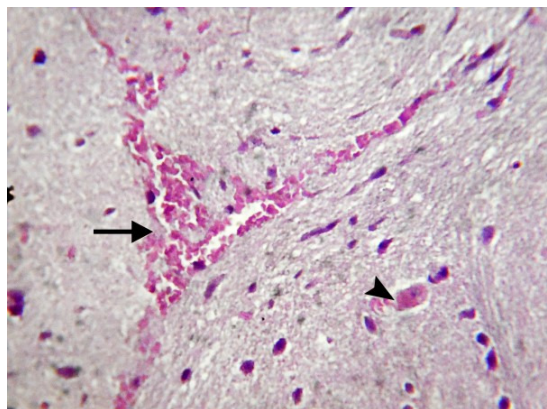**Brain of rat treated with, OLZ in electrocution group showing swollen and degenerated axon axon (arrow), HE x400**

Figure-18
Brain of rat treated with, CLZ in electrocution group showing parenchymal hemorrhage (arrow) and necrotic neuron (arrowhead), HE x400
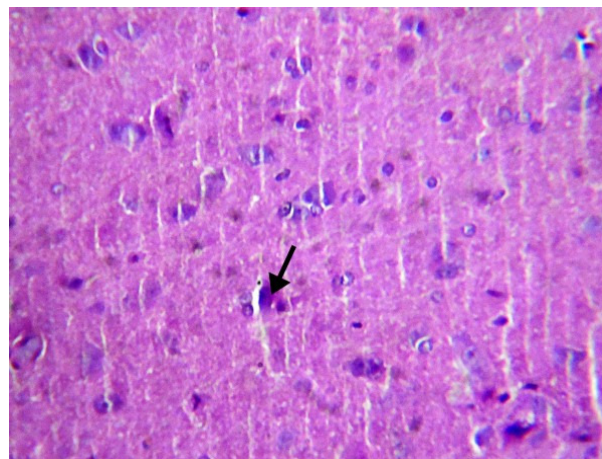

Figure-21
Brain of rat treated with, OLZ in starvation group showing degenerated neuron (arrow), HE x400
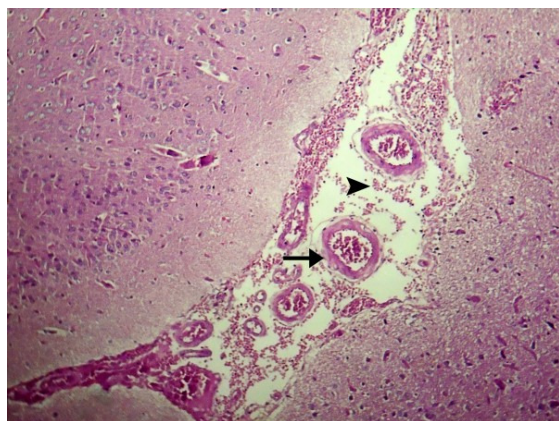

Figure-19
Brain of rat treated with, OLZ and CLZ in electrocution group showing slight congestion of cerebral blood vessels (arrow) with perivascular hemorrhage (arrowhead), HE x100
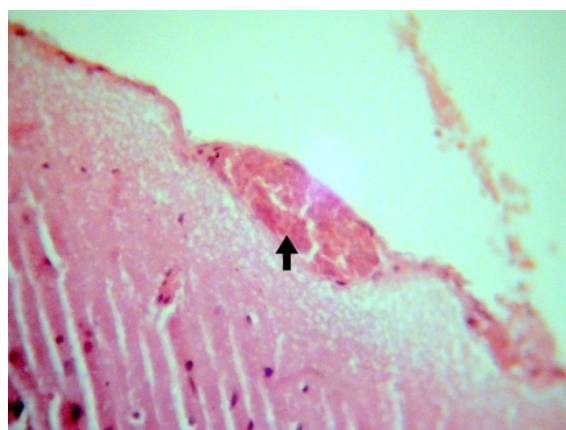
Figure-22
Brain of rat treated with, CLZ in starvation group showing parenchymal hemorrhage (arrow) and necrotic neuron (arrowhead), HE x400

Figure-20
Brain of control, untreated rat, in starvation group showing multiple pyknotic nuclei of the degenerated neurons (arrowheads) with marked vacuolation of the cerebral neuropil (asterisks), H&E X400

Figure-23
Brain of rat treated with, OLZ and CLZ in starvation group showing marked vacuolation of the neuropil (arrows) with presence of extravastaed erythrocytes (arrowheads), HE x400

Conclusion

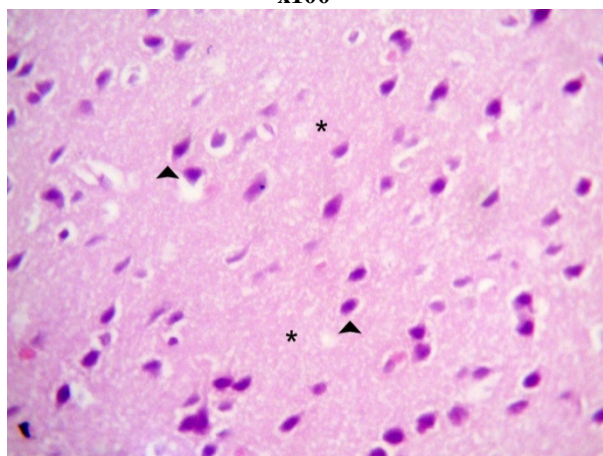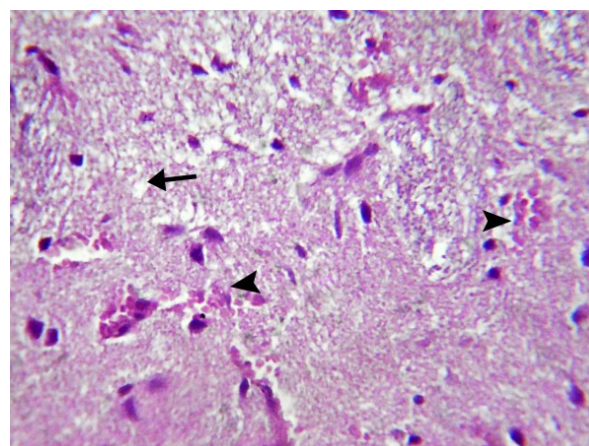In summary, the data of the current study provide an ample evidence via noticeable detection of Olanzapine and Clozapine residues in rat brain of adult male rats after applying different modes of death including; drowning electrocution, and starvation using HPLC-UV technique.

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