

RESEARCH ARTICLE

COMPARATIVE ALTERATIONS OF SOME BIOCHEMICAL PARAMETERS IN *RATTUS NORVEGICUS* RATS FOLLOWING FLUNITRAZEPAM ADMINISTRATION

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ABSTRACT: Background: Flunitrazepam is a class of benzodiazepine and a central nervous system depressant which is a sedative-hypnotic used in the treatment of anxiety, insomnia etc. This study was aimed at comparing the alterations of some biochemical parameters in *Rattus norvegicus* rats following flunitrazepam administration. **Methods:** Each of the seven rats in experimental group one and two respectively was administered orally with 0.8mg/1.24kg of flunitrazepam as a single dose for one day, experimental group one (acute toxicity study) and 0.2mg/1.24kg of flunitrazepam daily for a period of four weeks, experimental group two (sub-chronic toxicity study) while each of the other seven rats that were monitored as controls (control group) was not administered with flunitrazepam or any other drugs. Thereafter five milliliters blood specimen were collected into lithium heparin anticoagulated bottles and spun to obtain plasma samples that were used for the quantitative measurement of alanine aminotransferase, aspartate aminotransferase, C-reactive protein, urea and creatinine. **Results:** The results revealed statistically significant elevations ($p < 0.05$) in the mean values of all the measured biochemical parameters in both experimental group one and two rats respectively as compared with that of the control group. **Conclusion:** In conclusion, acute and sub-chronic administration of flunitrazepam may cause hepato-renal and inflammatory disorders in *Rattus norvegicus* rats. It is therefore recommended that further study on the histomorphological examination of hepato-renal organs be carried out in order to ascertain the true picture of damage on these organs in the rats.

KEYWORDS: Alterations of biochemical parameters, Flunitrazepam administration, *Rattus norvegicus*.

INTRODUCTION:

Flunitrazepam, with a trade name known as Rohypnol, is a central nervous system depressant in a class of drugs called benzodiazepines which are sedative-hypnotics used in the treatment of anxiety, insomnia, sleep disorder, seizure disorder and skeletal-muscle relaxants. This drug is commonly prescribed for anxiety and sleep disorders in Europe,

Latin America, and elsewhere, but not approved for use or sale in the United States. Examples of other benzo diazepam are alprazolam (xanax), bromazepam chlordiazepoxide (librium), lorazepam (atavan) and diazepam (valium), all of which are readily prescribed in the United States [1].

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Flunitrazepam drug contains one or two milligrams of *flunitrazepam* and are round, white, odourless, and tasteless, they dissolve undetected in liquid and are often smuggled into the United States, via international mail, and sold on the streets in the manufacturer's blister packaging similar to over-the-counter cold medicine or birth control pill packaging [1]. In response to reports that have implicated *flunitrazepam* in drug-facilitated sexual assaults, the drugs were reformulated by the manufacturer as oblong green composed of a dye that turns blue when dissolved in liquid, thus making it easier to detect in some drinks [2]. It is the third most prescribed sleeping medication worldwide which when taken alone are relatively safe drug. However, increasing dosages produce signs of progressive central nervous system and brain function depression besides more serious side effects such as hyperthermia, liver damage and kidney failure.

This drug is widely abused by many individuals including both young and old without the prescription of a physician. Given that the liver is an organ responsible for the metabolism of drugs and the kidneys responsible for the removal of waste products of metabolism couple with the fact that availability of data on the effects of *flunitrazepam* on biochemical parameters such as aspartate aminotransferase, alanine aminotransferase, C-reactive protein, urea and creatinine are rare, these biochemical parameters alanine aminotransferase, aspartate aminotransferase (liver enzymes), urea and creatinine (renal biomarkers) were considered vital to measure in *Rattus norvegicus* rats following the administration of *flunitrazepam* with the aim of assessing the adverse effects this drug may have on these organs. Besides, C-reactive protein was also measured in an attempt to assess inflammatory disorder that may arise during the course of *flunitrazepam* administration. These steps were taken in this present study with a view that the findings will give an insight on the likely adverse effects this drug may impose on humans.

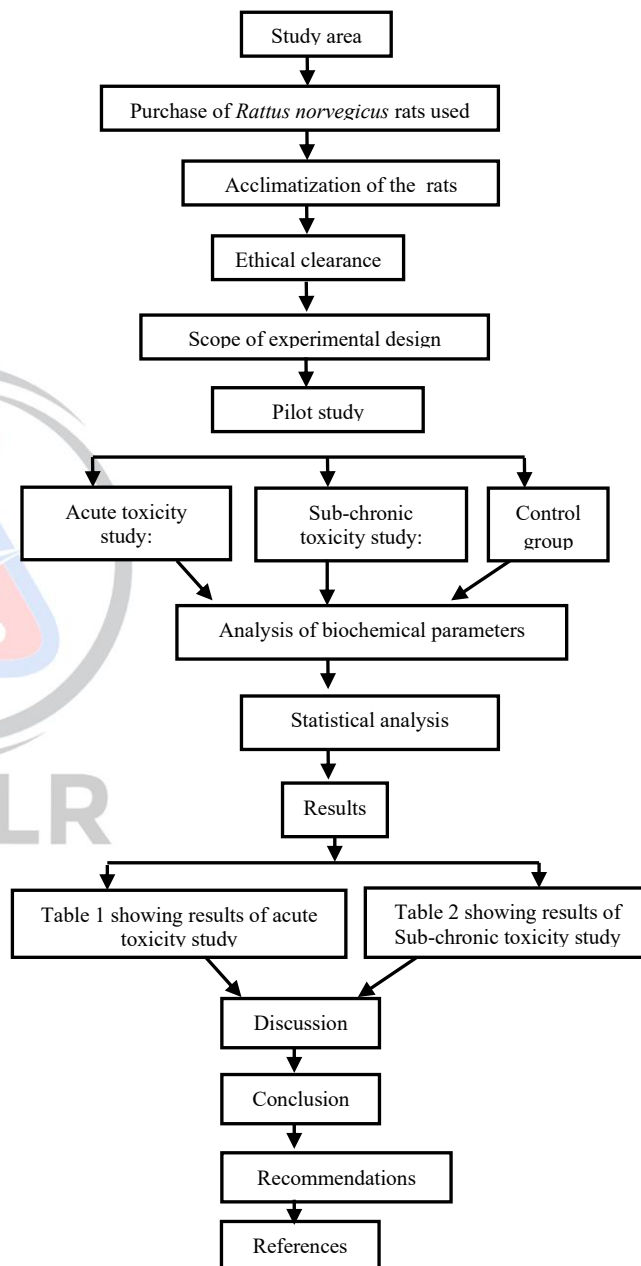
MATERIALS AND METHODS:

The schematic representation of workflow for this study is shown in Figure 1.

Study area

This study was carried out in the Department of Medical Laboratory Science, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

Figure 1; Diagram showing the schematic representation of workflow



Animals used

The male *Rattus norvegicus* rats used for this study were purchased from a reputable animal house in Yenagoa, Bayelsa State, Nigeria and transported to the animal house of the Department of Medical Laboratory Science, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria via a private transport. The rats were allowed to acclimatize in the animal house for two weeks and were observed for physical deformity or any ailments that may render them unfit prior to the commencement of the study. The rats which were placed in ventilated iron standard cages were fed with pre-mix rat feed and water *ad libitum*.

Ethical clearance

This study which got the ethical approval from the ethical committee of Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria was carried in conformity to the National Guidelines for Animal usage in research.

Scope of experimental design

The study was carried out on four months old male *Rattus norvegicus* rats with each of them within the weight range of 1.23 ± 0.05 kg and grouped as follows

- (i) Experimental group one: Each of the rats in this group which were used for acute toxicity study was administered orally with 0.8mg/1.24kg of *flunitrazepam* as a single dose for one day
- (ii) Experimental group two: Each of the rats in this group which were used for sub-chronic toxicity study was administered orally with 0.2mg/1.24kg dose of *flunitrazepam* daily for a period of four weeks
- (iii) Control group: Each of the rats in this group was not administered orally or otherwise with *flunitrazepam* or any other drugs before and during the course of this study

Reagents used

Commercially available alanine aminotransferase, aspartate aminotransferase, urea, creatinine (Randox Diagnostic kit, UK) and C-reactive protein (Spin-react Diagnostic kit, Spain) used for this research were purchased in Timako Medicals, Idumota, Lagos State, Nigeria. The manufacturers' standard

operational procedures were strictly adhered to while carrying out these tests.

Equipment used

Medical equipment and scientific Limited Vis-spectrophotometer with model number S23A13192 was used for absorbance measurement of the respective biochemical parameters. Other equipment and materials that were used include: Gulfex medical equipment and scientific Limited, macro-centrifuge with model number 800D, incubator with model number DNP-9052A, Refrigerator and automatic micropipettes

Pilot study

A pilot Study was carried out to ascertain the minimum dose of oral consumption of *flunitrazepam* that can cause 100% death (LD_{100}) and 50% death (LD_{50}) respectively in the experimental *Rattus norvegicus* rats

A total of 16 rats having approximately the same weight of 1.23 ± 0.05 kg were used. These rats which were grouped into 3 with 4 rats/group were designated as A, B and C respectively with each rat in group A administered with 0.8mg/1.24kg of *flunitrazepam* as a single dose, group B administered with 1.2mg/1.24kg of *flunitrazepam* as a single dose and group C administered with 1.8mg/1.24kg of *flunitrazepam* as a single dose respectively for one day and thereafter monitored for 24 hours for any signs and symptoms of *flunitrazepam* toxicity including death. The rats were considered dead when they failed to respond to agitation.

The following signs and symptoms such as convulsion, respiratory distress, coma and death occurred in all the rats in group C (LD_{100}) while the same signs and symptoms occurred in 50% of the rats in group B (LD_{50}). The LD_{50} was calculated according to the arithmetic method as described by [14] and was eventually rated according to the toxicity rating method as described by [15]

Acute toxicity study (Experimental group one)

In this study each of the seven rats was administered orally with 0.8mg/1.24kg of *flunitrazepam* drug as a single dose for a day. At the end of this, the rats were monitored for 24 hours for any signs or symptoms.

The following signs, weakness and itching were observed. After this the rats were anaesthetized using the inhaled chloroform technique with five milliliters blood specimens withdrawn from the cardiac of each and dispensed into lithium heparin anti-coagulated bottles respectively for biochemical investigations

Sub-chronic toxicity study (Experimental group two)

In this study each of the seven rats was administered orally with 0.2mg/1.24kg of *flunitrazepam* drug daily for a period of four weeks. At the end of this experiment, the rats were anaesthetized using the inhaled chloroform technique with five milliliters blood specimens withdrawn from the cardiac of each and dispensed into lithium heparin anti-coagulated bottles respectively for biochemical investigations

Control group

In this study another seven rats which were not administered orally or otherwise with *flunitrazepam* or any other drugs before and during the course of the study were monitored as controls.

Biochemical parameters analyzed with specified methods

The following biochemical parameters as shown below were analyzed.

- i. Alanine aminotransferase in accordance with the colorimetric method as described by ^[3] using reagents manufactured by Randox Laboratories, Limited, 55, Diamond Road, Crumlin, County, Antrim, BT294QY, United Kingdom.
- ii. Aspartate aminotransferase in accordance with the colorimetric method as described by ^[3] using reagents manufactured by Randox Laboratories, Limited, 55, Diamond Road, Crumlin, County, Antrim, BT294QY, United Kingdom.
- iii. Urea in accordance with Urease Berthelot method as described by ^[4] using reagents manufactured by Randox Laboratories, Limited, 55, Diamond Road, Crumlin, County, Antrim, BT294QY, United Kingdom.
- iv. Creatinine in accordance with Jaffe reaction method as described by ^[5] using reagents

manufactured by Randox Laboratories, Limited, 55, Diamond Road, Crumlin, County, Antrim, BT294QY, United Kingdom.

- v. C-reactive protein in accordance with latex turbidimetry method as described by ^[4] using reagent manufactured by Spin-react Diagnostic, Spain.

Statistical analysis

The results obtained from the control and experimental groups were expressed as mean and standard deviation while the differences between the groups were compared using the student's 't' test. A p-value of $p \leq 0.05$ was considered statistically significant.

RESULTS:

The results of the biochemical parameters measured in the control group and experimental group one (acute toxicity study) rats are shown in Table 1.

The results revealed that the mean values of plasma alanine aminotransferase, aspartate aminotransferase, C- reactive protein, urea, and creatinine were significantly elevated statistically ($p < 0.05$) in the *Rattus norvegicus* rats orally administered with 0.8mg/1.24kg of *flunitrazepam* as a single dose for one day as compared with the mean values of the control rats.

The results of the biochemical parameters measured in the control group and experimental group two (sub-chronic toxicity study) rats are shown in Table 2.

The results revealed that the mean values of plasma alanine aminotransferase, aspartate aminotransferase, C- reactive protein, urea and creatinine were significantly elevated statistically ($p < 0.05$) in the *Rattus norvegicus* rats orally administered with 0.2mg/1.24kg of *flunitrazepam* daily for a period of four weeks as compared with the mean values of the control rats.

A box-plot illustrating further the control, acute toxicity study and sub-chronic toxicity study data is shown in "Inserted Figure 2"

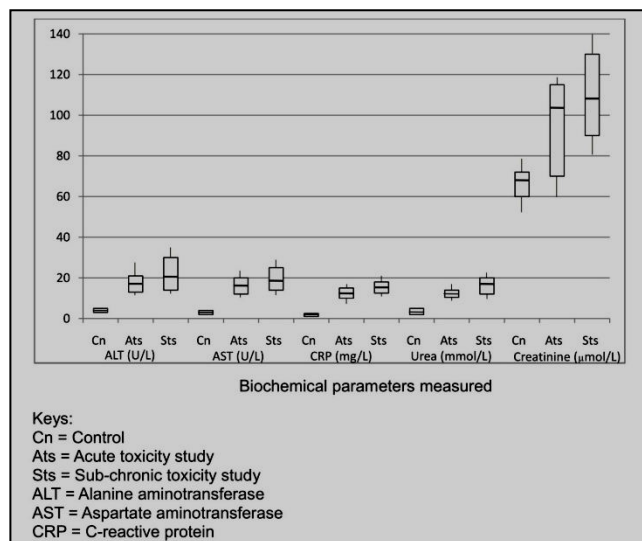


Figure 2: A box-plot showing data of control, acute toxicity study and sub-chronic toxicity study

DISCUSSION:

In this study comparison was made between the mean values of plasma alanine aminotransferase, aspartate aminotransferase, C-reactive protein, urea and creatinine in male *Rattus norvegicus* rats that were orally administered with 0.8mg/1.24kg of flunitrazepam as a single dose for one day, experimental group one (acute toxicity study) and another male *Rattus norvegicus* rats that were orally administered with 0.2mg/1.24kg of flunitrazepam daily for a period of four weeks, experimental group two (sub-chronic toxicity study) respectively with that of male *Rattus norvegicus* rats that were not orally administered with flunitrazepam or any other drugs, control group. This comparison was necessitated based on the indiscriminate higher dose consumption of flunitrazepam as a single dose by many individuals, particularly the young ones in the study community (acute toxicity study) as well as the indiscriminate routine consumption of this drug by individuals both young and old in the study community (sub-chronic toxicity study) without taking into consideration whether this act may have any adverse effects on their health.

Alanine aminotransferase is an aminotransferase enzyme that catalyzes the inter-conversion of amino acid to 2-oxo-acid by transfer of amino group from alanine to the alpha keto group of ketoglutaric acid to

generate pyruvic acid [6]. This enzyme which is clinically measured as part of liver function test [7] and more hepatocellular specific than aspartate aminotransferase [8] was firstly characterized by [9] in the mid-1950s.

The results from this study as shown in “Inserted Table 1” revealed statistically significant elevation ($p < 0.05$) in the mean value of plasma alanine aminotransferase in the experimental group one rats (acute toxicity study) as compared with that of the control group. This finding which is established in this study is presumed to be linked with hepatocellular injury which had led to the leakage and subsequent release of this enzyme from the liver to the plasma following the administration of 0.8mg/1.24kg of flunitrazepam.

Aspartate aminotransferase is an aminotransferase enzyme that catalyzes the inter-conversion of amino acids to 2-oxo-acids by transfer of amino group from aspartate to the alpha keto group of ketoglutaric acid to generate oxaloacetic acid [6]. It is found in all tissues except bone [10].

The results from this study as shown in Table 1. revealed statistically significant elevation ($p < 0.05$) in the mean value of aspartate aminotransferase in the experimental group one rats (acute toxicity study) as compared with that of the control group which also may be indicative of hepatocellular injury thus leading to the leakage and subsequent release of this enzyme from the liver to the plasma following the administration of 0.8mg/1.24kg of flunitrazepam. as established in this study.

C-reactive protein is a pentraxin family of protein which derived its name from its ability to react with C-polysaccharides isolated from pneumococcal cell walls. The production rate of C-reactive protein increases in inflammation, infection, cystitis or bronchitis, this rate falls drastically once these causative factors subside, this however, is as a result of its short half-life [11].

The results from this study as shown in Table 1. revealed that the mean value of plasma C-reactive protein was significantly elevated statistically ($p < 0.05$) in the male *Rattus norvegicus* rats in experimental group one (acute toxicity study) as compared with that of the control group. This finding is presumed to be

due to systemic inflammation following the oral administration of 0.8mg/1.24kg of *flunitrazepam* which had led to the release of interleukin-6 and other cytokines with the subsequent increase in synthesis of C-reactive protein as established in this study.

Urea is the main nitrogenous breakdown product of protein metabolism in mammals which is excreted in urine with more than 90% of its industrial production worldwide used as a nitrogen release fertilizer [12].

The results in this study as shown in Table 1, went further to reveal statistically significant increase ($p<0.05$) in the mean value of urea in plasma of *Rattus norvegicus* rats in experimental group one, acute toxicity study following the administration of 0.8mg/1.24kg of *flunitrazepam* when compared to that of the control group. This finding as established in this study may be suggestive of renal impairment.

Table 1: Biochemical parameters measured in the control group and experimental group one (acute toxicity study) rats

Parameters	Control group (n=7)	Experimental group (n=7)	p-value	Remark
ALT (U/L)	4.52 ± 0.31	18.00 ± 1.87	$p<0.05$	S
AST (U/L)	3.84 ± 0.25	16.83 ± 1.24	$p<0.05$	S
CRP (mg/L)	1.84 ± 0.43	12.67 ± 1.06	$p<0.05$	S
Urea (mmol/L)	3.57 ± 1.06	12.43 ± 3.61	$p<0.05$	S
Creatinine (mmol/L)	66.75 ± 3.46	103.17 ± 4.57	$p<0.05$	S

Values are expressed as means ± SD **KEYS:** ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRP = C-reactive protein, S = statistically significant, n = number of rats

Creatinine is a nitrogenous waste product that is derived from creatine and creatine phosphate. It is not reutilized, but is excreted from the body in the urine via the kidney with its serum level increasing slightly significant 24 hours after exercise [13]. As a consequence of the way in which it is excreted by the kidney, its measurement is used almost exclusively in the assessment of kidney function.

The results from this study as shown in Table 1. revealed that the mean value of plasma creatinine was significantly higher statistically ($p<0.05$) in the male *Rattus norvegicus* rats in experimental group one, acute toxicity study following the administration of 0.8mg/1.24kg of *flunitrazepam*.when compared to that of the control group. This finding as established in this study may be suggestive of renal impairment.

As shown in Table 2, the results went further to revealed more statistically significant elevations ($p<0.05$) in the mean values of all the measured plasma biochemical parameters in the male *Rattus norvegicus* rats that were administered orally with 0.2mg/1.24kg of *flunitrazepam*, experimental group two, sub-chronic toxicity study. These findings as established in this study may serve as a pointer to the fact that sub-chronic oral administration of 0.2mg/1.24kg of *flunitrazepam* daily for a period of four weeks may also result in hepato-renal and inflammatory disorder in *Rattus norvegicus* rats due to injury imposed on the liver, adverse effects on the kidneys and the release of interleukin-6 and other cytokines respectively.

Table 2: Biochemical parameters measured in the control group and experimental group two (sub-chronic toxicity study) rats

Parameters	Control group (n=7)	Experimental group (n=7)	p-value	Remark
ALT (U/L)	4.52 ± 0.31	22.00 ± 1.96	$p<0.05$	S
AST (U/L)	3.84 ± 0.25	19.29 ± 1.72	$p<0.05$	S
CRP (mg/L)	1.84 ± 0.43	15.50 ± 1.41	$p<0.05$	S
Urea (mmol/L)	3.57 ± 1.06	16.14 ± 3.72	$p<0.05$	S
Creatinine (mmol/L)	66.75 ± 3.46	110.57 ± 4.81	$p<0.05$	S

Values are expressed as means ± SD, **KEYS** ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRP = C-reactive protein, S = statistically significant, n = number of rats

CONCLUSION:

This study has revealed that the plasma levels of alanine aminotransferase, aspartate aminotransferase, C - reactive protein, urea and creatinine are altered in *Rattus norvegicus* rats

administered orally with 0.8mg/1.24kg of flunitrazepam as a single dose for one day (acute toxicity study) as well as 0.2mg/1.24kg of flunitrazepam routinely (daily) for a period of four weeks (sub-chronic toxicity study) respectively which may be indicative of hepato-renal and inflammatory disorders

RECOMMENDATIONS:

(i) Flunitrazepam administration should strictly be on the prescription of a physician or healthcare professional as its indiscriminate use may impose adverse effects on humans.

(ii) Routine biochemical profile of patients and/or individuals on this medication should be monitored regularly.

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