

## Effects of acute and chronic Triazolam treatments on brain GABA levels in albino rats

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**Abstract.** The present study investigated the effects of acute and chronic intraperitoneal administration of Triazolam on g-aminobutyric acid (GABA) levels in different brain areas of albino rats. Three experiments were conducted. In the first, five groups of rats were acutely treated with different doses of Triazolam (0.25 mg/kg-4.0 mg/kg). In the second experiment, rats were treated chronically with a single daily dose of Triazolam (started with 0.25 mg/kg and increased by time to 1.0 mg/kg) for 5 weeks, simulating clinical use. In the third, rats were treated chronically with three daily doses of Triazolam (started with 0.25 mg/kg and increased by time to 0.5 mg/kg) for 20 days, representing a form of drug abuse. Brain levels of GABA and plasma levels of Triazolam were measured using high performance liquid chromatography (HPLC). The acute Triazolam administration produced an increase in GABA levels in all brain areas studied. The chronic administration of single daily dose of Triazolam produced normal GABA levels in all brain areas except brain stem where the levels were significantly decreased; this indicates the development of tolerance to Triazolam action on increasing GABA content. The chronic administration of three daily doses of Triazolam produced a decrease in GABA levels in all brain regions studied. In conclusion, chronic single daily dose treatment (representing normal use) produces tolerance to Triazolam effects on brain GABA levels, while chronic three daily doses administration (akin to drug abuse) causes a fall in GABA levels.

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**Key words:** Albino rats, drug abuse, GABA, HPLC, tolerance, triazolam

## INTRODUCTION

Systemically administered benzodiazepines (BZs) exert their effects by potentiating GABAergic transmission through their interaction at the benzodiazepine receptor on the BZ-GABA receptor complex (Haefely 1978, Koe 1979, Paul et al. 1981). Acutely administered benzodiazepines selectively enhance postsynaptic GABAergic neurotransmission and increase GABAergic responsiveness (Saad 1972, Gonsalves and Gallager 1985). This was due to GABA formation being increased by BZs *via* stimulation of glutamate decarboxylation (Saad et al. 1995). It was also reported that full and selective allosteric modulators, like diazepam, can maximize GABA action with therapeutic doses (0.25 mg/kg) (Costa and Guidotti 1996).

The prolonged exposure to benzodiazepines markedly attenuates postsynaptic GABAergic neurotransmission and reduces the magnitude of potentiation to only 4% (Gonsalves and Gallager 1985). A structural shift in GABA<sub>A</sub> receptors is produced by the chronic administration of full allosteric modulators. This mechanism might account for the appearance of tolerance and withdrawal symptoms associated with long-term treatment with BZs (Costa and Guidotti 1996). However the studies on the effect of Triazolam on GABA levels in different brain regions after acute and chronic administration are almost nonexistent. Therefore, the aim of this work is to study the acute effect of Triazolam at different dosage regimes on GABA levels in various brain regions, and to investigate the possible development of tolerance to chronic administration of Triazolam and its relation to brain GABA levels.

## METHODS

### Animals

Male Albino Wistar rats weighing between 150 and 250 gms, bred in the animal house of the Al-Fateh Medical University, were used. Each group was housed separately in a cage (L: 60cm, W: 38cm, H: 20 cm), except during measurements. Standard rat food pellet diet and water were available *ad libitum*. The animals were kept at constant room temperature (20-25°C), and on a 12 h dark/light cycle.

### Drugs and administration

Triazolam (TZ) was supplied by Upjohn Company, Egypt; the drug was administered intraperitoneally as suspension in 1% Tween 80 (T80) in water (Collinge et al. 1983). The drug suspension was administered with a constant volume of 1.0ml/kg of body weight (Assandri et al. 1984).

### Procedure

#### ACUTE ADMINISTRATION

The rats were divided into six groups of 10 animals each. Group I. The control group received only a single dose of 1% T80. Group II received a single dose of 0.25 mg/kg TZ. Group III received a single dose of 0.5 mg/kg TZ. Group IV received a single dose of 1.0 mg/kg TZ. Group V received a single dose of 2.0 mg/kg TZ. Group VI received a single dose of 4.0 mg/kg TZ. In all the groups, brain homogenates were prepared for GABA measurement immediately after one hour of Triazolam or T80 administration.

#### CHRONIC ADMINISTRATION (SINGLE DOSE PER DAY)

Fifteen male Albino Wistar rats were divided into two groups. Each group was housed separately in a cage. Group I. The control group ( $n = 5$ ) received a single daily dose of 1% T80 for five weeks. In Group II. Triazolam was administered in an ascending dose for five weeks; treatment started with a daily dose of 0.25 mg/kg for two weeks, increased to 0.5 mg/kg for the following two weeks, and finally to 1.0mg/kg for the fifth week ( $n = 10$ ). In both groups, the brain homogenates were prepared for GABA measurement one hour after the last dose of T80 in group (I), or of Triazolam in group (II).

#### CHRONIC ADMINISTRATION (THREE DOSES PER DAY)

Male Albino Wistar rats were divided into two groups of 10 animals each. Each group was housed separately in a cage. Group I, the control group, received three daily doses of 1% T80 for 20 days. Group II received three daily doses of Triazolam for 20 days. Triazolam was administered in an ascending dose starting with three daily

doses of 0.25 mg/kg for ten days, which was then increased to 0.5 mg/kg for the following ten days.

In both groups, the brain homogenates were prepared for GABA measurement one hour after the last dose of T80 for group (I) or 12 h after the last dose of TZ for group (II).

### Biochemical assay for measuring GABA level

Animals were killed by cervical dislocation and the body was exposed to a microwave irradiation for 4 s (Schmid and Karobath 1977, Zecca et al. 1982, Saller and Czupryna 1989). The brain was rapidly removed and the cerebellum, the brain stem, the striatum, the cerebral cortex, and the mid-brain were dissected on an ice-cold petri dish. The tissues were placed after weighing in pre-cooled 100 ml plastic tubes. Ice-cooled 0.1M perchloric acid (10 ml) which contained Valine (internal standard) at a concentration of 15 µg/ml was added to the tissue. The tissues were homogenized for one minute during which the tube was embedded in an ice bath, then centrifuged at 5,000 rpm for 10 min at 4°C. The supernatants were stored at -20°C until assayed.

Dansylation reaction was induced using the method of Saller and Czupryna (1989). Dansylation was carried out by adding 100 µl of each supernatant of the samples or the standards to a micro-tube containing 100 µl of 0.1M potassium carbonate solution. These solutions were mixed using vortex and then centrifuged using micro-centrifuge at 10,000 rpm for 10 min. One hundred µl of each supernatant was transferred into a pyrex tube containing 100 µl of 0.1 M sodium hydrogen carbonate solution, to which 400 µl of working dansyl chloride solution (1.25 mg/ml anhydrous acetone) was added. The tubes were shaken for 30 s using vortex and then incubated at 90°C in benchtop oven for 30 min. The tubes were not capped during the incubation, to allow most of the solvent to be evaporated. This did not appear to adversely affect the progress of the dansylation reaction and served to concentrate the samples. After getting the tubes out of the oven, they were left to cool down to room temperature, and the dansylated derivatives were transferred to 1.5 ml microtubes and stored at -20°C until assayed.

C8 reversed-phase HPLC columns (5 µm, 250 x 3.2 mm) were used to resolve and quantify the samples (Zecca et al. 1982). The HPLC mobile phase (Saller and Czupryna 1989) consisted of a deionized helium degassed water-acetonitrile (HPLC grade) mixture (65:35,

v/v) containing 0.15% (v/v) phosphoric acid. The flow rate was kept at 0.5 ml/min. The detector excitation was at 333 nm and emission at 532 nm.

Twenty five µl of the dansyl derivative of the GABA samples were transferred to HPLC micro-sample vials and injected into the column. Retention time of GABA and internal standard were found to be in the range of 4.96 and 5.85 min respectively. The peak ratios of the samples were calculated with reference to the internal standard.

GABA levels were expressed for the different discrete brain regions as µg/gm of tissue. The total values were summed up and recalculated for the total weight of the discrete regions combined (whole brain) and expressed as µg/gm of the total weight.

### Measurements of plasma levels of Triazolam

The treated animals were killed by cervical dislocation and then rapidly decapitated; the body was held upside-down, and the blood was collected in oxalate fluoride tubes. The blood was stirred and then centrifuged at 3,000 rpm for five minutes. Plasma was separated and frozen until assayed. Plasma samples for Triazolam assay were prepared by adding 0.1 ml of plasma of the treated rats to a tube containing 0.1 ml of internal standard diazepam solution (of 1.0 µg/ml methanol) and 0.2 ml methanol.

The standard solutions were prepared by adding ascending concentrations of Triazolam to plasma obtained from the control group of 1% T80 treated rats, and processed with the same technique as applied to the treated rats.

The mixtures of samples or standards were mixed using a vortex for 20 s then centrifuged using a micro-centrifuge for five minutes. The volume of 25 µl of the supernatant was injected into HPLC (Abdel-Hamid and Abuirjeie 1988).

The HPLC system consisted of a variable wavelength monitor (2151 LKB Bromma) and a reversed-phase column (LKB, Lichrosorb RP18, 5 µm, 250 x 4 mm) from Bromma, Sweden. The mobile phase was composed of a water : methanol : acetonitrile (30 : 63 : 7) mixture. The flow rate was 1ml/minute. Spectrophotometric wavelength was 221 nm. for Triazolam (Schmith 1991).

### Statistical analysis

Linear regression was applied for the standard solutions peak ratio from which the concentration of the sam-

ples as calculated using Microsoft Excel - version 7 software.

Descriptive statistical analysis was applied on parameters of different samples using the NCSS software to verify whether the data were normally distributed using Kolmogorov-Smirnov maximum deviation test for goodness of fit. If the parameters were normally distributed, treatments were compared by one-way ANOVA. If the parameters were not normally distributed, treatments were compared by the Mann-Whitney two samples (non-matched) test. The differences were considered significant at  $P < 0.05$ .

## RESULTS

### Acute Triazolam administration

The results and the statistical analysis for the changes in GABA concentration in different brain areas after acute administration of TZ are given in Table I.

The acute administration of TZ produced significant increase in GABA content in the striatum for all doses (0.25 mg/kg-4 mg/kg), with no significant difference between the effects of the different doses. Brain stem GABA content also increased significantly especially with the low dose (0.25 mg/kg) of TZ. The GABA content of cerebellum, cerebral cortex and whole brain was

increased significantly at 0.25 mg/kg to 2.0 mg/kg doses of Triazolam. The highest dose (4 mg/kg) however did not produce any significant change in GABA content in these areas of brain. In case of mid-brain, lower doses (0.25 mg/kg and 0.5 mg/kg) produced a significant increase in GABA content while higher doses (1.0 mg/kg-4 mg/kg) had insignificant effects as compared to controls. In all brain areas with the exception of the striatum and brain stem, the highest dose (4 mg/kg) did not produce any significant change in GABA content as compared to the control group. In general the brain GABA content, with the exception of the striatum, was significantly lower with the 4 mg/kg TZ dose when compared with smaller doses (0.25, 0.5 mg/kg doses). The effect of the 2 mg/kg dose was also like that of 4 mg/kg in all areas of brain except for cerebellum, cerebral cortex and whole brain.

### Chronic Triazolam administration (single dose per day)

The chronic administration of a single dose of Triazolam did not change GABA levels in discrete brain regions and in the brain in toto when compared with control group treated with T80, except for that in brain stem where GABA levels were decreased significantly (Table IIA).

Table I

Effect of acute administration of Triazolam on GABA content in different brain regions						
Treatments	Cerebellum	Brain stem	Striatum	Cerebral cortex	Mid-brain	Whole brain
T80 ml/kg (control)	150.48 ± 4.98	120.32 ± 3.80	448.84 ± 14.12	170.30 ± 6.82	235.03 ± 6.54	181.53 ± 5.81
0.25 mg/kg TZ	202.49 ± 4.79*	152.28 ± 3.87*	664.80 ± 25.43*	210.95 ± 4.44*	257.59 ± 4.23*	220.63 ± 2.17*
0.5 mg/kg TZ	179.15 ± 4.69 <sup>*,a</sup>	140.36 ± 4.72 <sup>*,a</sup>	684.38 ± 55.92*	199.98 ± 4.13*	259.16 ± 3.81*	213.65 ± 2.79*
1.0 mg/kg TZ	175.76 ± 3.87 <sup>*,a</sup>	141.13 ± 4.33*	625.80 ± 37.79*	198.10 ± 3.77 <sup>*,a</sup>	245.07 ± 3.61 <sup>a,b</sup>	220.52 ± 12.92*
2.0 mg/kg TZ	182.91 ± 5.13 <sup>*,a</sup>	147.21 ± 6.27*	636.10 ± 34.69*	189.45 ± 3.96 <sup>*,a</sup>	241.74 ± 7.08 <sup>b</sup>	205.23 ± 3.18 <sup>*,a</sup>
4.0 mg/kg TZ	160.39 ± 2.99 <sup>a,b,c,d</sup>	137.24 ± 3.05 <sup>*,a</sup>	675.70 ± 54.58*	179.16 ± 4.25 <sup>a,b,c</sup>	213.89 ± 5.66 <sup>a,b</sup>	192.55 ± 3.57 <sup>a,b,c,d</sup>

The values are the means (µg/gm tissue) ± SE of GABA for 10 rats; \*, significantly different from the control T80 treated group  $P < 0.05$ ; a, significantly different from the 0.25 mg/kg treated group  $P < 0.05$ ; b, significantly different from the 0.5 mg/kg treated group  $P < 0.05$ ; c, significantly different from the 1.0 mg/kg treated group  $P < 0.05$ ; d, significantly different from the 2.0 mg/kg treated group  $P < 0.05$ .

Table II

Effect of chronic administration of Triazolam on GABA content in different brain regions						
Treatments	Cerebellum	Brain stem	Striatum	Cerebral cortex	Mid-brain	Whole brain
<b>A</b>						
Group I (control)	144.06 ± 10.96	121.88 ± 13.63	442.29 ± 16.62	161.7 ± 11.28	228.62 ± 14.04	176.14 ± 13.68
T80 (chronic)	(5)	(5)	(5)	(5)	(5)	(5)
Group II	139.32 ± 8.74	92.89 ± 5.10*	456.30 ± 23.99	141.39 ± 11.76	235.26 ± 13.69	172.11 ± 14.24
TZ (chronic)	(9)	(10)	(10)	(9)	(10)	(9)
<b>B</b>						
Group I (chronic)	169.90 ± 6.85	159.30 ± 5.23	374.90 ± 7.51	170.20 ± 5.33	220.40 ± 3.73	181.30 ± 2.34
T80 (chronic)	(9)	(9)	(9)	(9)	(9)	(9)
Group II	95.80 ± 3.11*	91.70 ± 5.77*	349.50 ± 7.26*	97.70 ± 6.31*	182.60 ± 10.25*	118.60 ± 4.20*
TZ (chronic)	(9)	(9)	(9)	(9)	(9)	(9)

The values are the means ( $\mu\text{g/gm}$  tissue)  $\pm$ SE for the number of observations shown in parentheses; A, TZ doses (once daily) started from 0.25 mg/kg and increased by time up to 1.0 mg/kg during 5 weeks; B, TZ doses (three doses per day) started from 0.25 mg/kg and increased by time up to 0.5 mg/kg during 20 days; \*, Significantly different from control (group I) at  $P < 0.05$ .

### Chronic Triazolam administration (three doses per day)

The chronic administration of three daily doses of Triazolam lowered significantly whole brain levels of GABA when compared with the control group treated with T80. This effect was found in all parts of the brain studied: cerebellum, brain stem, striatum, cerebral cortex and midbrain (Table IIB).

### Triazolam concentrations in plasma

The plasma concentration of Triazolam after acute administration of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg doses were found to be  $0.278 \pm 0.011$ ,  $0.353 \pm 0.006$ ,  $0.440 \pm 0.011$ ,  $0.473 \pm 0.010$  and  $0.554 \pm 0.011$   $\mu\text{g/ml}$  respectively.

The plasma concentration of Triazolam after chronic administration of a single daily dose was found to be  $0.379 \pm 0.030$   $\mu\text{g/ml}$ . It was  $2.830 \pm 0.110$   $\mu\text{g/ml}$  12 h after the last dose of chronic three daily dose administration.

## DISCUSSION

The acute administration of TZ produced increased GABA levels with the lowest dose (0.25 mg/kg) in different brain areas and when the whole brain was analysed (Table I). The increase in GABA concentration might be due to the fact that BZ increases GABA synthesis through the stimulation of glutamate decarboxylation (Saad et al. 1995). The therapeutic effects of BZs might be due to the increase in GABA levels in the regions where the specific binding sites in cerebellum, cerebral cortex, the limbic system and reticular formation are present (Potokar and Nutt 1994). The other possibility for BZ action may be through full selective allosteric modulation maximizing GABA action (Giusti et al. 1993, Costa and Guidotti 1996). The modulator allosteric site on the GABA-Chloride channel complex mediates both facilitatory (BZ-receptor agonists) and inhibitory (inverse agonists) effects (Haefely 1990). Other mechanisms involve neither GABA nor alterations in membrane permeability to chloride ions (Polc 1988, Burt and Kamatchi 1991, Brown and Bristow 1996,

Collado et al. 1998). The action of TZ on GABA may be mediated through the endogenous neurosteroids. It has been shown that activation of mitochondrial and glial BZ receptors (Krueger and Papadopoulos 1992) and stress (Purdy et al. 1991) increase neurosteroidal biosynthesis. These neurosteroids, including derivatives of progesterone, stimulate GABA and its neurotransmitter mechanisms (Majewska et al. 1986, Morrow et al. 1990, Puia et al. 1990). The endogenous neurosteroids may also contribute to the stimulation of GABA-ergic mechanisms and anxiolysis (Reddy and Kulkarni 1997a,b, Wieland et al. 1997, Rodgers and Johnson 1998).

Increased doses of TZ maintained the raised GABA content in almost all the areas of brain though the increase was significantly lower as compared to the low dose (0.25 mg/kg) of TZ except in striatum (Table I). It appears that the low dose of TZ used in this study had the maximal effect on brain GABA levels. Further increase in dose therefore could not penetrate the ceiling effect of TZ on GABA levels probably through GAD stimulation and other mechanisms. This view is substantiated by a report (Choi et al. 1981) which mentions that at saturating agonist concentrations, BZs have no enhancing effect on GABA levels. The statistically significant lower GABA levels in all brain regions except in striatum with higher doses of TZ as compared to the lowest dose is intriguing. It may be speculated that the higher brain concentration of BZ with higher doses might have increased the conversion of GABA recognition sites to a higher affinity form, and maybe to a maximum (Otero Losada 1988, 1989), with the exclusion of the feedback inhibition of GABA biosynthesis, involving the rate-limiting GAD enzyme. This continued persistence of the operation of a self-regulatory inhibitory effect on the rate-limiting enzyme would decrease the GABA levels with the higher doses of TZ. In the case of striatum, probably the ceiling point was not reached even at high TZ dose. The stimulant action on GABA levels therefore persisted.

Chronic administration of a single daily dose of TZ produced no significant change in brain GABA levels (Table IIA). This indicates the development of tolerance to TZ action on increasing GABA content, as compared with acute TZ administration. The lack of significant TZ effect may be related to the short duration TZ pharmacokinetic profile, combined with the decrease in number of BZ receptors induced by BZs with short half-life (Scharf and Feile 1983, File 1985). It has also been shown that chronic flurazepam administration decreases

efficacy of BZ agonists and that the effect is reversed after 72 h withdrawal (Hu and Ticku 1994). It is possible that tolerance developed to the GAD-stimulating action of TZ without a significant change in brain GABA levels. On the other hand, there was a significant decrease in the brain stem.

There are reports of changes in biochemical and physiological properties of tissues in chronically tolerant animals. There is mention of decreased expression of the gene encoding GABA receptors in tolerant animals (Heninger et al. 1990, Zhao et al. 1994). There was also a decrease in mRNA coding of the receptor's  $\alpha 1$  sub-unit in frontoparietal cortex and hippocampus, but not in sensory cortex, striatum and cerebellum. The activity of the sub-unit becomes normal after 72 h of withdrawal (Impagnatiello et al. 1996). In the present study, the single TZ dose chronic regimen does not show any significant change in brain GABA levels except in the brain stem where there was a significant decrease.

The development of tolerance to a drug has been explained from different viewpoints: feed-back increased enzyme synthesis of a related neurotransmitter for counter-adaptation (Goldstein and Goldstein 1968, Shuster 1971); activation of redundant pathways (Martin 1968); decreased neuronal activity causing disuse supersensitivity (Jaffe and Sharpless 1968); increased receptor population of counterbalancing neurotransmitters (Collier 1966); down regulation of BZ receptors (Cumin et al. 1982); desensitization of GABA receptor (Cash et al. 1997); or decrease in enhancement of BZ binding without changes in sub-unit gene expression (Pratt et al. 1998).

One might speculate that the activity of neurotransmitter systems like catecholamines, GABA and glycine with their well-documented inhibitory effects might be decreased during tolerance to CNS inhibitory drugs. This is understandable in the wake of the negative feed-back control of neuronal activity by recurrent neural pathways, alteration of the activity of regulatory macromolecules (receptors) and metabolic pathways of neurochemicals leading to the latent counter-adaptation forming the basis of the development of tolerance. These complex changes may be "within-system adaptations" and "between-system adaptations" (Koob and Bloom 1988). Therefore, decrease in GABA levels with chronic administration as compared to acute TZ administration is expected, though those found were not significantly different from the control values. This might be due to the complex inter-relationship between different brain

regions and neurochemicals as mentioned above. However, the significant decrease of GABA levels in brain stem may be related to the latent CNS effects which may take place after withdrawal. This view is substantiated by a report which mentions decreased GABA levels during withdrawal syndrome of another short-acting BZ-lorazepam (Saad et al. 1995). The lorazepam withdrawal in mice was shown to result in the up-regulation and binding of the drug to the GABA-A receptor complex (Miller et al. 1988).

The above picture is quite different when TZ is administered chronically in three daily doses (Table IIB). In this case, there was a precipitous and severe fall of GABA levels in all the brain regions in sharp contrast to the single daily dose of TZ treatment, even though the plasma TZ levels were sharply increased. One is confronted with the transition from increased brain-GABA levels with acute TZ administration to normal levels with chronic one dose daily treatment to a precipitous and severe fall in GABA levels with the chronic three doses daily administration.

Several mechanisms have been described to explain the development of tolerance to the receptor-mediated drug actions. These include attenuation of the signals from the stimulated receptors involving covalent modification (homologous desensitization), destruction of receptors, and feed-back regulation of receptors leading to down-regulation (Perkins et al. 1991, Nowakowska et al. 1997). The observed decrease in GABA concentrations in brain with multiple daily doses might be related to the neuroadaptive mechanisms regulating brain neurotransmitters (Edwards et al. 1981). These mechanisms are probably as a result or the cause of the development of complete tolerance. It is well known that the continued presence of a ligand near receptors leads to desensitization or down-regulation of receptors. This is also true for chronic administration of BZs, which leads to a desensitized and non-functional form of receptor (Gonsalves and Gallagher 1985) resulting in pharmacodynamic tolerance (Tietz et al. 1986, Miller et al. 1987, 1988). The same phenomenon is also expected to occur to BZ receptor related GABA biosynthesis through stimulation of GAD. The chronic administration of TZ will result in down regulation of BZ receptors which are responsible for the stimulation of GAD enzyme. This could explain the normal GABA levels seen with one-dose daily chronic administration of TZ. On the other hand, a significant fall in GABA levels with the three daily dose schedule might be due to severe desensitization of BZ re-

ceptor in relation to GAD stimulated biosynthesis of GABA. Another possible facet may be the effect of chronic administration of TZ producing initial GABA release leading to final depletion (Hitchcott et al. 1990).

In conclusion, acute administration of TZ resulted in an increase in GABA levels in all the brain regions studied. It is important to emphasize here that the smallest dose (0.25 mg/kg) had the ceiling effect on brain GABA levels. Chronic once daily administration resulted in tolerance to GABA elevating effects while chronic administration with repeated daily doses produced a fall in GABA levels.

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*Received 4 November 1999, accepted 28 June 2000*