

Evaluation of the pathophysiological effects of bromazepam on some clinical chemistry parameters of rat

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ABSTRACT

The main aim of this study is to evaluate the pathophysiological effects of using high doses (single and repeated) of bromazepam, a broad-spectrum sedative, on blood glutathione (GSH), lipid peroxidation (TBA) and some clinical chemistry parameters of rat. Rats were orally supplemented with a single dose (4.5 mg/kg B.wt) or repeated doses (4.5 mg/kg B.wt for 7 consecutive days) of bromazepam. Another two sets of animals were orally administered with the same volume of the vehicle (distilled water) and considered as control groups. Blood samples were collected at different time points during the first 24 hours after the single dose treatment and for 10 days following the last treatment in case of repetitive dose administration to monitor the changes in the tested parameters. Blood GSH was significantly decreased after administration of the drug, while levels of serum TBA were significantly increased. Serum total protein values were elevated during the whole time course, but this was not significant. Meanwhile, creatinine, urea, uric acid and the activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) were significantly increased as a result of treatment with either dose. We conclude that using high doses of bromazepam significantly alters both liver and kidney functions, and that GSH might play an important role in the elimination of these side effects. Bromazepam increased lipid peroxidations in rat tissues by a mechanism dependent on glutathione levels during the 24 hr. post-treatment. A clear tendency for a positive correlation between bromazepam concentration and serum lipid peroxidation levels was recorded. It is advisable that people who take high doses of this drug, should be aware of these effects, especially those suffering from liver and kidney problems.

KEYWORDS: Bromazepam, glutathione, lipid peroxidation, clinical chemistry, rat.

INTRODUCTION

Anxiety and sleep disorders are the most common problems of the recent years and people used to take anxiolytic drugs to over-come these problems. Most of these drugs affect neurohumoral transmission by interfering with the neurotransmitters, blocking the receptors, and hence inhibiting their action (Arnett & Ritchie 1961). Bromazepam is a member of the benzodiazepines group, widely used as an anxiolytic, sedative and hypnotic drug. It is absorbed completely through the gastrointestinal tract and its peak plasma concentration is reached within 1-4 hours after oral administration. The plasma elimination half-life ranges from 7.9 to 19.3 hours. Bromazepam is basic in nature and about 70% of it is bound to plasma proteins (Neumeyer & Booth 1995). It is metabolized in the liver into active metabolites which are excreted entirely as glucoside conjugates of 3-hydroxybromazepam (27%), 3-hydroxybenzoylpyridine derivatives (40%), intact bromazepam (2.3%) and intact benzoylpyridines derivatives (0.66%).

Remy *et al.* (1995) described a case of cross hepatotoxicity and liver dysfunction for patients treated with tricyclic antidepressants including bromazepam; the levels of serum

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aspartate aminotransferase (AST) and alanine aminotransferase (ALT) returned to normal when the drug was withdrawn. They suggest that the chemical moiety, the tricyclic ring, might be involved in the hepatotoxicity. Some patients suffered from bromazepam intoxication when serum levels reached 6mg/l, which induced central respiratory arrest and defects in the brain-stem reflexes. Without intensive medical care the patients would not have survived (Rudolf, *et al.* 1998). Another important finding was reported by Kudo *et al.* (1997) after the death of a patient as a result of taking large number of sleeping pills. They found high levels of some psychotropic drugs in his blood, including bromazepam. This lethal combination, which had been prescribed to treat his depression, was apparently the main cause of his death.

Assignment of a particular compound of the sedative-hypnotic class of drugs indicates that its major therapeutic use is to cause sedation with concomitant relief of anxiety or to encourage sleep. These clinical uses are of such magnitude that sedative-hypnotics are among the most frequently prescribed drugs worldwide (Trevor & Way 1987). Depending on the dose, these drugs produce sedation, hypnosis, anesthesia, coma, and death. The exact mechanism by which they produce psychic and physical dependence is unclear (Hollister 1987). No one knows how many persons are dependent on sedatives. Much of the presumed dependence is psychological, possibly based on the need for these drugs as a form of replacement therapy. During recent years, much interest has developed in the phenomenon of “therapeutic dose dependence”. In addition, several patterns of sedative abuse have emerged. People with severe emotional disorders may use these drugs to seek escape into oblivion. Most abusers of sedatives use them to produce an altered mental state with dis-inhibition in the same way that many people, or they themselves, use alcohol.

The present investigations aimed to evaluate the pathophysiological effects of using high doses (single and repeated) of bromazepam on the liver and kidneys of the rat. Because of their importance as indicators for the stability of the biological system against the metabolic reactions that generate reactive oxygen species, glutathione (GSH) and lipid peroxidation (TBA) were evaluated.

MATERIALS AND METHODS

Drugs, doses and chemicals: Bromazepam (Calmepam[®]) tablets (3mg concentration) were obtained from Amoun Pharmaceutical Industries Co (Cairo, Egypt). And suspended in distilled water (1 mg bromazepam/1 ml distilled water). Animals were administered the drug orally by a gastric tube in a dose equal to 4.5 mg/kg b.wt. as a single dose or as a repeated dose for 7 consecutive days.

All the chemicals used in the present, study including glacial metaphosphoric acid, ethelene di-amine tetra acetic acid (EDTA), NaCl, sodium monophosphate, 5-5’ dithiobis-2-nitrobenzoic acid (DTNB), sodium citrate, phosphoric acid and butanol, were purchased from Sigma Chemical Company (St. Louis, USA).

Experimental animals: Male Sprague-Dawely rats (175-200 g) were obtained from the breeding unit of the National Research Center, Dokki, Giza, Egypt. Rats were housed five per cage in a well-ventilated room (25 ± 2°C) and 12 hours light/dark cycle at the animal house of the Zoology Department, Suez Canal University, Ismailia, Egypt. Animals were regularly fed on a standard diet *ad libitum*. Rats were divided into four groups (10 rats/group). In the first group rats were orally administered a single dose of bromazepam (4.5 mg/kg b.wt.). The second group was orally treated with 4.5 ml-distilled water/kg b.wt. (vehicle) and considered as a control group. Animals of the third group were given repeated doses of bromazepam (4.5 mg/kg b.wt/day for 7

consecutive days). The accumulated dose for this group after the last treatment was 31.5 mg/kg b.wt. The last group were orally supplemented with the same volume of the vehicle as the pervious group and considered as a control group.

Experimentation: Blood samples were collected from the first two groups (single dose treatment) after 2, 4, 8, 12, 16, 20 and 24 hours post-treatment and from groups 3 and 4 after 1, 3, 5, 7, 9 and 11 days from the last administration. Collections of blood were made using orbital sinus technique (Sanford 1954). Heparinized whole blood was used to determine glutathione (GSH) according to the method of Beutler *et al.* (1963). To obtain serum, the collected blood was incubated at 37°C for 5 minutes in clean dry test tubes, then centrifuged at 3000 rpm for 20 min. Sera were aspirated and stored at -20°C until used. Serum was used in the determination of the levels of lipid peroxidation, total proteins, creatinine, urea and uric acid, as well as, in the determination of the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Determination of the levels of lipid peroxidation in serum was based on the measurement of thiobarbituric acid (TBA) reactants following the technique of Sharma & Wadhwa (1983). The other clinical chemistry parameters were measured using kits obtained from BioMerieux kits (Laboratory Reagent and Instrument, France). A UV-visible spectrophotometer (Shimadzu-1601-PC, Japan) was used in the present study.

Statistical analysis: The effect of bromazepam was determined by comparing each of the two groups (control vs. treated) using Student's unpaired t-test (Snedecor 1956). Group differences were considered statistically significant at the level of $P < 0.05$.

RESULTS

Blood glutathione (GSH) content in both the control and the treated groups with a single oral administration of bromazepam (4.5 mg/kg) is graphically demonstrated in Figure 1 (A). Blood GSH content was observed to exhibit significant decreases of 12.5, 21.8, 21.7 and 21.9 % after 12, 16, 20 and 24 hrs respectively. The effect of repeated oral administration of bromazepam (4.5 mg/kg for 7 days) on GSH content in both control and treated groups is shown in Figure 1 (B). Blood GSH levels decreased significantly after 1, 3, 5 and 7 days post-last treatment as compared to their control values. The maximum decrease was recorded in the first three days following drug withdrawal. However, it was noticed that blood GSH levels returned back to approximately the same values as the control animals after 9 and 11 days.

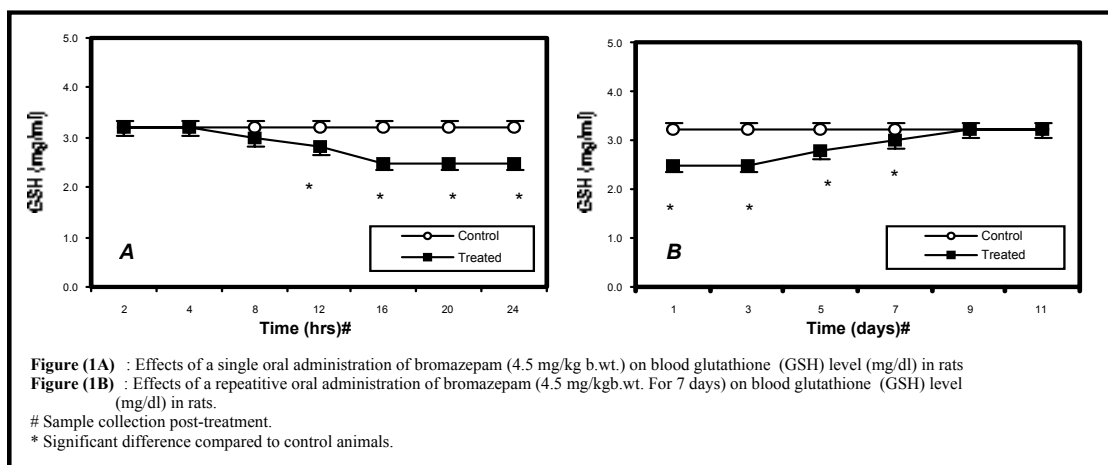
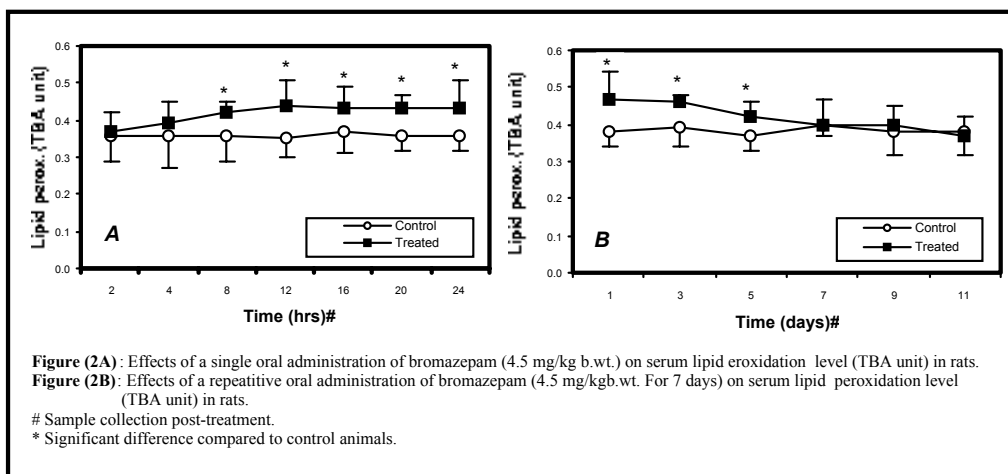
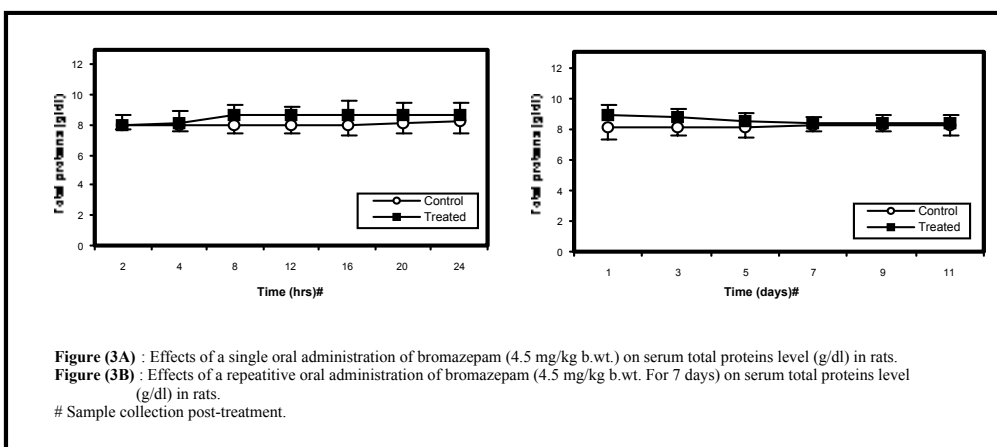


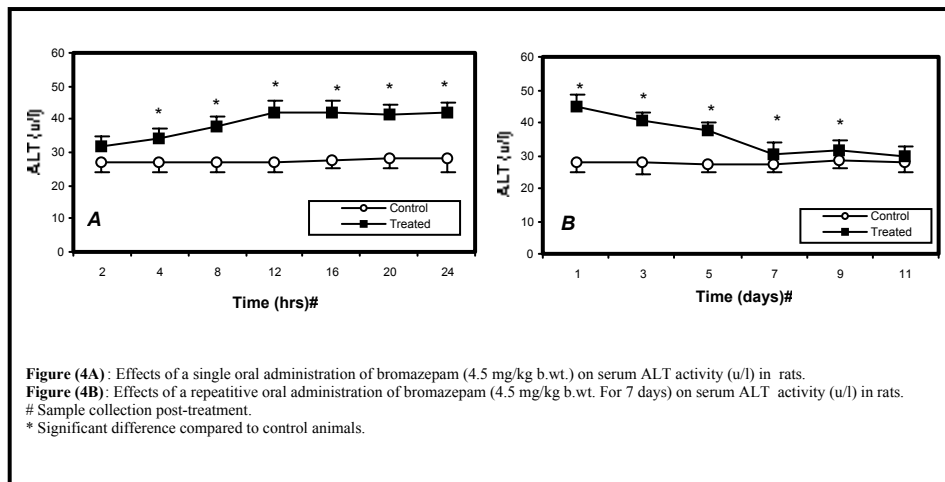
Figure 2 (A) illustrates the effects of a single dose of bromazepam on serum lipid peroxidation (TBA levels) in rats. After 8, 12, 16, 20 and 24 hrs of bromazepam treatment, serum lipid peroxidation was significantly elevated and the highest increase was 29% observed after 12 hours. The same results were obtained following repeated-dose administration, where serum lipid peroxidation significantly increased by 23.8, 17.9 and 13.5% after 1, 3 and 5 days post-treatment of bromazepam respectively. Lipid peroxidation levels returned back to almost the same value as the control 7 days after the last treatment (Figure 2B).



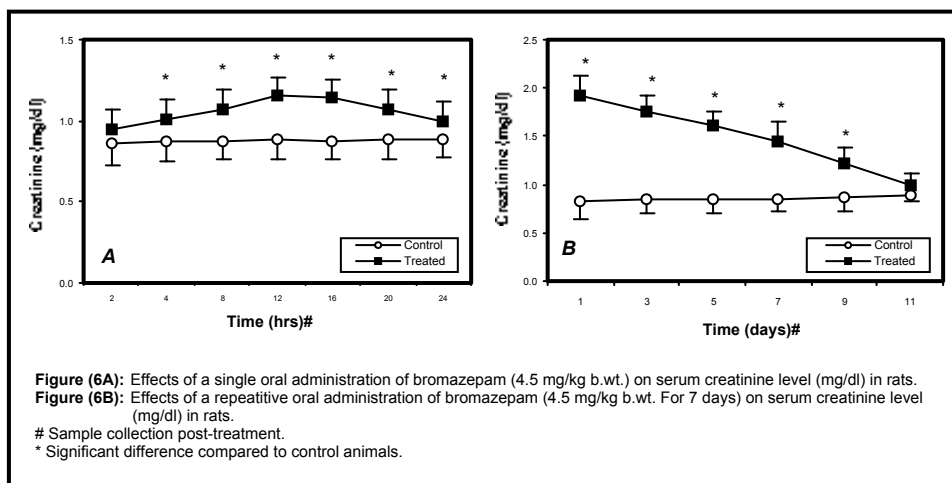
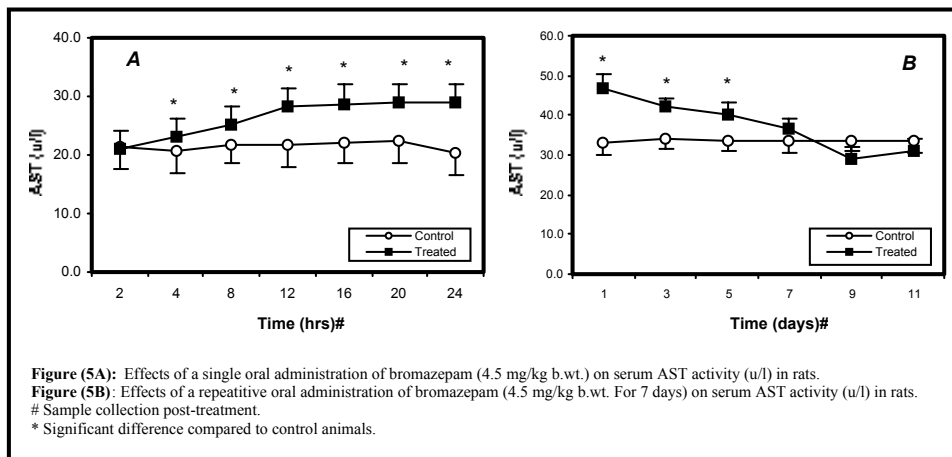
The level of serum total proteins in control and bromazepam single and repeated dose-treated rats at different time intervals are illustrated in Figures 3 (A&B). Although levels of serum total proteins increased slightly, there were no statistical differences compared to the control values.



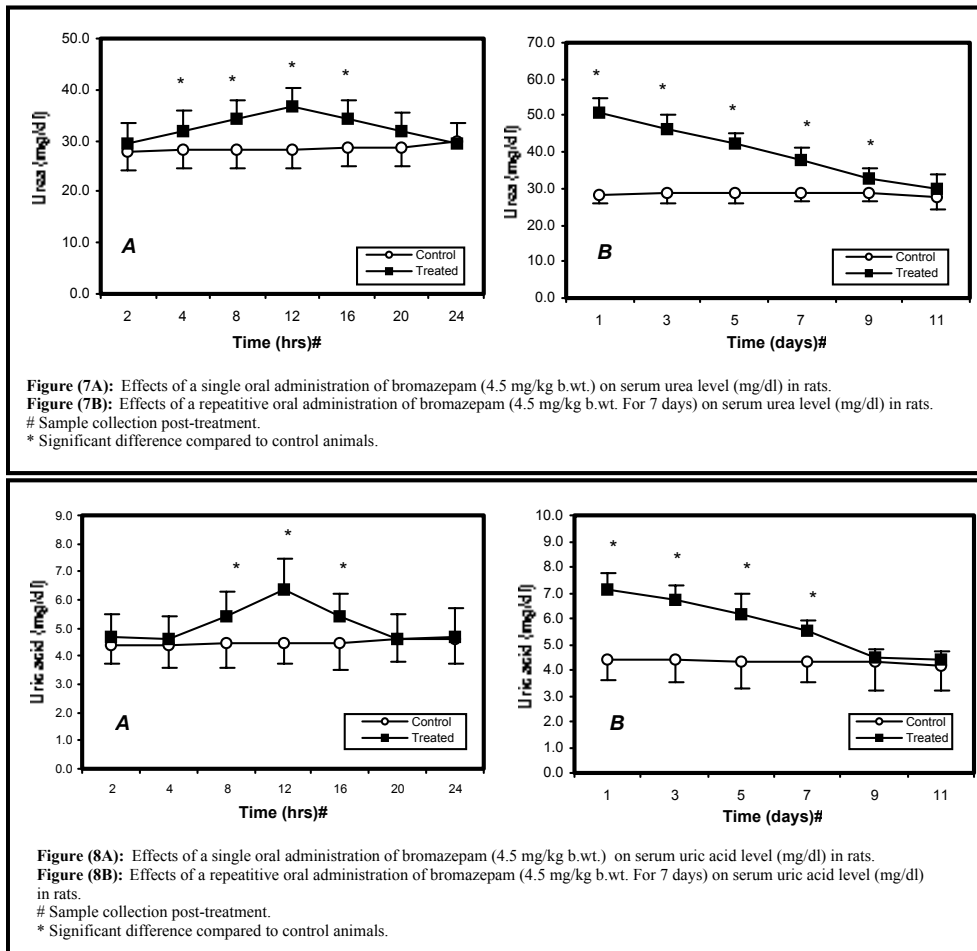
Activities of serum ALT in bromazepam single dose-treated animals is shown in Figure 4 (A). Level of serum ALT significantly increased during the whole experiment as compared to their corresponding controls. Again, serum ALT levels significantly increased in the case of repeated-dose administration (Figure 4B). The maximum peak was recorded in the first 24 hours after stopping the treatment and returned to the control value after 11 days post-last treatment.



Results in Figure 5 (A) demonstrate the effect of single-dose administration of bromazepam on the activity of serum AST in rats. Serum AST values were significantly increased with values equal to 11.9%, 16.2%, 31.5%, 29.7%, 29.6% and 42.4% after 4, 8, 12, 16, 20 and 24 hrs post-treatment respectively, as compared to their corresponding controls. Repeated doses of bromazepam elevated serum AST levels. A significant increase was observed after 1, 3 and 5 days post-last treatment (Figure 5B). However, AST levels had returned to the control value 7 days after the withdrawal of the drug.



Creatinine levels in the serum of bromazepam single dose-treated rats were significantly increased by 16.1%, 22.9%, 30.6%, 31.0% and 21.6% after 4, 8, 12, 16 and 20 hrs post-treatment respectively, relative to controls (Figure 6A), and the same patterns were evident following repeated-dose administration (Figure 6B). It was observed that the creatinine level reached its maximum value (132.5%) one-day after the last treatment and returned to the same level as the controls after 11 days.



Urea levels were observed to exhibit significant increases of 12.8%, 22.0%, 30.1% and 20.4% after 4, 8, 12 and 16 hrs post-treatment respectively as a result of the single-dose treatment with bromazepam (Figure 7A). Repeated doses of the drug induced significant increases in the serum urea levels (Figure 7B) after 1, 3, 5, 7 and 9 days post-last treatment with levels returning to the control value at the end of the experiment. Up 16 hrs after a single-dose of bromazepam, serum uric acid levels were significantly increased (Figure 8A). In case of repetitive treatment, the optimum significant increase (61.4%) in serum uric acid values was recorded after the first day from drug withdrawal (Figure 8B).

DISCUSSION

The current era of benzodiazepines began in 1960. In the opinion of many authorities these drugs are now the sedative-hypnotics of therapeutic choice. Abuse and physical dependence have been reported in some cases. The term “drug abuse” is unfortunate because it connotes social

disapproval and may have different meanings to different people. Abuse of a drug might be construed as any use of a drug for non-medical purposes, almost always for altering consciousness. Another important issue is related to the same concept, which is drug misuse. Misusing a drug means that it might be taken for the wrong reason, in the wrong dosage, or for too long a period (Hollister 1987). The present study was undertaken to investigate the pathophysiological effects of high doses (single and repeated oral administrations) of bromazepam on blood glutathione (GSH), lipid peroxidation (TBA), total proteins, ALT, AST, creatinine, urea and uric acid levels in rat serum.

GSH is a tripeptide of cystine, glutamic acid and glycine, and is the principal nonprotein-sulfhydryl compound in the tissue. In addition to being a major cofactor for GSH peroxidase, GSH is necessary for the stability of sulfhydryl-containing enzymes and protects hemoglobin and many other cofactors from oxidation (Jocelyn 1972). It is present in the liver at a concentration of about 5mM (Gilette *et al.* 1974). The high levels of GSH in many animal and plant cells suggest that it may have important biological functions since it participates in a variety of biosynthetic and detoxification reactions (Jocelyn 1972). The toxicities of numerous chemicals are associated with the depletion of hepatocyte GSH or may become enhanced after depletion of GSH by pretreatment with other xenobiotics (Abd El-Rahman *et al.* 1999). GSH plays a critical role in many cellular processes, including the metabolism and detoxification of oxidants, metals and other reactive electrophilic compounds of both endogenous and exogenous origin (Lee *et al.* 1998). The protective role of GSH against the effect of bromazepam has not been discussed before. In the present study, results showed a significant decrease in blood GSH content occurred after 12 to 24 hours after bromazepam administration in the case of single dose, and during the first 7 days after the stoppage of the repeated doses. Depletion of blood glutathione may be due to a nucleophilic scavenger of the reactive metabolites of bromazepam in hepatic cells. Ballatori & Rebbor (1998) mentioned that the liver is the major site of GSH synthesis and GSH is released at high rates into both blood plasma and bile. Hepatic metabolism accounts for the clearance or elimination of all benzodiazepines. The two major pathways involved are microsomal oxidation, including N-dealkylation or aliphatic hydroxylation, and subsequent conjugation by glucuronyl transferases to form glucuronides that are excreted in the urine. One important feature of benzodiazepines metabolism is the formation of active metabolites with effects on the central nervous system, some of which may be long-lived (Trevor & Way 1987). Those benzodiazepines for which either the parent drug or active metabolites have long half-lives are more likely to cause cumulative effects with multiple doses (Danneberg & Weber 1983). GSH levels were returned back to the control values after 9 and 11 days after withdrawal of the drug (repetitive treatment). This could be attributed to the capability of mammalian cells to resynthesize GSH after its depletion (Lertratanangkoon *et al.* 1997). The depletion in glutathione content may alter many important functions, e.g. maintenance of SH groups of proteins and small molecules, membrane and cellular integrity and biosynthetic activity in cells through the regulation of enzyme activity as well as the synthesis of macromolecules (Orsilles & Depaoli 1998).

Biological materials, particularly membranes, contain high concentrations of unsaturated lipids and in the presence of a free-radical initiator plus oxygen, they may be oxidized. This process, known as lipid peroxidation, has been implicated as a general biological degenerative reaction (Pla & Whecht 1976). As shown in the present study there was a significant increase in the level of hepatic lipid peroxidation up to 24 hours after bromazepam administration in the case of a single dose, and up to 5 days post-treatment in the case of repeated doses. This increase may

be due to the depletion of the blood glutathione content during these times. Glutathione depletion renders the animal more susceptible to free radical-mediated damage, especially damage induced by cellular lipid peroxidation (Konings 1986; Burton & Traber 1990). It has been observed that glutathione depletion is accompanied by an increase in the amount of TBA reactants in experimental animals (Moustafa 1998).

ALT and AST are intracellular enzymes and very low concentrations are found in serum. Both enzymes are widely distributed in the body tissues and are found in particularly high concentrations in those tissues with high metabolic activity. They are found mostly but not exclusively in the liver, heart, kidney and skeletal muscle tissues. Ingestion of high doses of bromazepam induced significant increases in the activity of serum ALT and AST compared to the control values. This increase could be ascribed to a mild damage in the hepatocyte plasma membrane as a result of the oxidative stress occurring during biotransformation of the high doses of bromazepam that cause more release of cytoplasmic ALT and AST into the serum (Nalpas *et al.* 1986). On the other hand, it was observed that the concentrations of ALT and AST returned to the control range after 9 days following bromazepam withdrawal, which suggests that the effects of this drug on the liver and other tissues are temporary and transient. This finding was in agreement with Davis *et al.* (1989).

The effect of bromazepam on the level of creatinine, urea and uric acid were evaluated in this investigation. These compounds are the most abundant nonprotein nitrogen constituents in the body and their determinations are the most commonly ordered tests of the kidney's ability to excrete metabolic wastes (Tresseler 1988). The data showed a significant increase in the levels of these compounds in the case of both single and repeated-dose treatments. Since increases in these values are used as indicators of renal failure, it can be postulated that high concentrations of bromazepam are closely related to the impairment of renal function.

In conclusion, using high doses of bromazepam significantly alters both liver and kidney functions and that GSH might play an important role in the elimination of these side effects. Bromazepam increased lipid peroxidations in rat tissues by a mechanism dependent on glutathione levels during the 24 hr. post-treatment period. A clear tendency for a positive correlation between bromazepam concentration and serum lipid peroxidation levels was recorded. It is advisable that people who take high doses of this drug accidentally or on purpose, should be aware of these effects, especially those suffering from liver and kidney problems.

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تقليل بعض الأعراض الجانبية لهذا العقار. وكذلك يمكن أن ننصح من يستخدم هذا العقار بجرعات عالية أن يحتاط من هذه الأعراض الجانبية وخاصة من يعانون من متاعب بالكبد أو الكلى.