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Toxicity Assessment of Benzalkonium Chloride and Dibromo Nitrilopropionamide in Wistar Rats

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Abstract: *Background:* Biocides are compounds that could be obtained from natural sources or through chemical synthesis. They used to control or prevent harmful organisms. They contain active ingredients such as microorganisms and substances that affect harmful organisms. Although low-concentration biocides have been used to control pests, they can be harmful more than industrial chemicals because humans are directly and frequently exposed to such biocides. Benzoalconium chloride (BAC) and dibromo nitrilepropionamide (DBNPA) are two biocides which are frequently used in the water treatment industry. Therefore, risk assessment of them are quite urgent. Hence, in this study, we investigate safety and potential toxicity of these biocides. *Methods:* The animals were randomly divided into 15 groups of five. Seven groups related to BAC and seven groups related to DBNPA, which received separately doses of 50, 300, 500, 1000, 2000, 3000 and 5000 mg / kg body weight (mg / kg), as they were treated orally (gavage) every 48 hours for two weeks; so that each group received a total of seven doses, respectively. Biochemical markers and hematological parameters were evaluated for toxicity assessment of BAC and DBNPA in female wistar rats. *Results:* Our findings demonstrated that the toxicity of DBNPA was more than BAC. The safe concentration was obtained 175 and 75.5 mg/kg in water for BAC and DBNPA, respectively. LD50 for BAC was 2346 mg / kg body weight (mg / kg), and for the DBNPA was 1062 mg / kg body weight (mg / kg). Lymphocyte level was significantly raised in groups that were treated with high doses of DBNPA. Other hematological parameters were not different significantly in both groups. In addition, there were no significant changes in biochemical parameters in both groups. *Conclusion:* To be conclude, BAC and DBNPA are safe and using them with standard concentrations in water treatment industries does not pose a problem for human health.

Keywords: Benzalkonium Chloride, Dibromo Nitrilopropionamide, Ld50, Toxicity, Rat

1. Introduction

Benzalkonium chloride has been known as methyl benzylammonium chloride which is a cationic surfactant. It is an organic salt that is classified as a quaternary ammonium compound. It is used in two main categories as a biocide, a cationic surfactant, and an ADBAC phase transfer agent [1]. BAC is readily soluble in ethanol and acetone and dissolution in water is slow. Concentrated solutions have a bitter taste and a faint odor like almonds. During COVID-19 epidemics, there is an occasional shortage of ethanol or isopropanol (as active ingredients) hand cleansers. The FDA has stated that BAC is eligible for use as a formulation in the hand rubbing formula of healthcare personnel [1, 2]. BAC are known as skin irritants; occasional reports are less common as allergens. In the case of acute toxicology data, BAC is classified by the EPA as toxic group II through oral and inhalation routes and group III toxicity through the dermal route. They are also considered to be very irritating to the eyes and skin (toxic group I) [3]. Small but significant genotoxic effects were observed in the human respiratory epithelial BEAS-2B cells *in vitro* condition for BAC. *In vitro* cell toxicity significantly was observed for BEAS-2B cells which are exposed to BAC different concentrations [4]. DBNPA is a fast biocidal agent which is easily hydrolyzed under both acidic and alkaline conditions

due to its instability in water as rapidly degrades and then decomposes to form a number of products depending on the conditions including ammonia, bromide ions, dibromoacetonitrile and dibromoacetic acid. It acts like ordinary halogen biocides and it is used in a wide range of applications. There are some examples of making paper as a preservative in paper coatings and grouts. It is also used as sludge control on machines and as a biocidal in hydraulic failure wells and in cooling water [5]. DBNPA has also been used as an antimicrobial agent in various industries. It is a fast-acting non-oxidizing biocide which is widely used in disinfection and control of biofilm formation especially in membrane processes such as reverse osmosis, nanofiltration, ultrafiltration and etc [5]. However, although several previous studies on the toxicity of BAC and DBNPA have been performed, there is a lack of toxicity studies on systemic toxicity of them in laboratory animals. Therefore, in the present study, the aim to assess toxicity of these biocides in rats. To the best of our knowledge, this the first safety study on BAC and DBNPA which are produced in Iran.

2. Materials and Methods

Materials: The amount of one liter was prepared from two commercial substances, BAC and DBNPA with the brand name of Zephiran Company (Iran). blood collection tubes containing K3-EDTA anticoagulant brand BD Vacutainer® (made in the USA) for hematological analysis. Hematology analyzer (Sysmex) made in Japan. German brand (Rotexmedica) ketamine and xylazine were used to anesthetize animals.

Animal experiment: In this experimental study, 75 adult female wistar rats with an age range of 8-12 weeks and a weight range of 170-200 g were purchased from the Animal Center and the experiments were performed at Faculty of Veterinary Medicine, University of Tehran. Throughout the experimental period, the animals were exposed to a light period of 12 hours of darkness and 12 hours of light and placed at room temperature of 23 °C, all of which had free access to drinking water and pellet food. All experiments were performed according to the ethical principles of working with laboratory animals, which was approved by the Faculty of Veterinary Medicine, University of Tehran (approval No of 7506023.6.17). The animals were randomly divided into 15 groups containing 5 animals. Group 1: Negative control (physiological serum 0.9%) at 5 ml / kg body weight received and seven groups related to the composition of BAC and seven groups of five related to the composition of DBNPA. They received doses of 50, 300, 500, 1000, 2000, 3000 and 5000 mg / kg body weight separately, respectively, every forty-eight hours for two weeks. The animals were gavaged so that each group would receive a total of seven doses. Groups of completely extinct animals were used only to calculate LD50, and other groups were used for biochemical and hematological experiments. Possible mortality was recorded daily, and 14 days after the first dose, the animals were morally euthanized and blood samples were taken.

Hematological parameters: Blood samples were taken from the heart and approximately 5 cc of blood samples from each animal were divided into two parts. A portion of the blood was transferred to the blood collection tubes containing K3-EDTA anticoagulant brand BD Vacutainer® (made in the USA) for hematological analysis. The second part of the blood sample was transferred into a 2 ml sterile Ependorf tube to prepare the serum. To prepare the serum, the samples were first centrifuged at 3000 rpm for 15 minutes. Then, the separated serum was removed inside the tubes with sufficient care and without disturbance and transferred to another 2 ml Eppendorf tubes. Blood counts was measured in whole blood, including hemoglobin (HGB), hematocrit (HCT), white blood cell count (WBC), red blood cell count (RBC), mean globular hemoglobin (MCH) mean globular volume (MCV), mean globular hemoglobin concentration (MCHC) and platelet count (PLT) as well as differential white blood cell count including POLY and LYM. These indicators were performed in the whole blood containing anticoagulants by a hematology analyzer (Sysmex) made in Japan.

Biochemical analysis: Serum biochemical markers include cholesterol (CHO), triglyceride (TG), high-density lipoprotein (HDL, LDL), and liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT). Alkaline phosphatase (ALP), and bilirubin (BILI) as well as creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)) using biochemical autoanalyzer (BT 3000, BioTechnica) made in Italy and using Pars test kits Made in Iran.

Statistical analysis: Results were analyzed as mean ± standard deviation of mean (Khomarlou, et al.). Statistics were achieved using SPSS Software (IBM, v.22; CA, USA). The differences between groups were evaluated by variance analysis (one-way ANOVA, Tukey post hoc test). P value<0.05 was statistically considered significant.

3. Results

LD 50 analysis: LD50 for BAC was 2346 mg / kg body weight (mg / kg), and for the DBNPA was 1062 mg / kg body weight (mg / kg). In the research conducted by Mr Handule Lee in 2019, the amount LD50 for benzoalkonium chloride in the laboratory animal Balb C241.7mg/kg has been calculated.

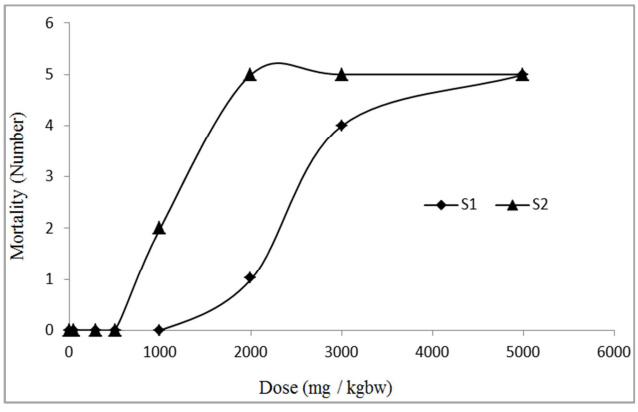

Figure 1. *The number of mortality obtained relative to the dose of compounds benzalkonium chloride (S1) and bromo nitrile propionamide (S2).*

The effect of biocides on hematological parameters: In this study, hematological parameters showed no significant differences in treated groups compared to the control group for DBNPA except in the parameter of lymphocytes, which the change was significant ($p < 0.05$). No significant change was observed for blood parameters in the case of BAC compared to the control group ($p < 0.05$).

Table 1. *Biochemical parameters of benzalkonium chloride and dibromonitrile propionamide groups in comparison with control group.*

Parameter	Unit	Control	S1				S2				
			50	300	2000	Sd	50	300	500	1000	Sd
CHO	Mg/dl	79.8	58.2	65.6	65.3	8-11	64	74.8	75	83.5	2-12
T. G	Mg/dl	255.4	101.4	119	88.3	15-70	90.6	144.6	436.5	316.5	33-61
HDL	Mg/dl	40.8	31.56	34.96	31.8	3-5	32	36.84	40.05	39.15	3-4
LDL	Mg/dl	26.98	21.76	22.82	26.8	3-5	26.18	26.46	24.3	29.45	2-5
SGOT	U/L	220	167	184.4	148.3	20-42	163.8	241.6	108	129	7-66
SGPT	U/l	100	69.6	61	49.0	7-13	61.2	74.4	44	55.5	3-25
ALK	U/l	393.6	427.2	375.4	426.7	67-228	373	276	402.5	333	113-143
BILI-T	Mg/dl	0.226	0.098	0.24	0.1	0.02-0.32	0.118	0.098	0.05	0.11	0.01-0.05
BILI-D	Mg/dl	0.044	0.046	0.044	0.1	0.01-0.02	0.054	0.06	0.015	0.07	0.01-0.03
CPK	U/l	892.2	856.4	825.2	517.3	86-269	631.2	767.2	503.5	551	53-424
LDH	U/l	2226.2	2178.8	2153.2	1699.7	391-756	1698.4	1636	982	1369	294-916

The effect of biocides on biochemical markers: Regarding the biochemical parameters, no significant difference was observed, except for the TG in the case of DBNPA and BAC compared to the control group ($p < 0.05$).

Table 2. *Hematology parameters of benzalkonium chloride and dibromonitrile propionamide groups in comparison with control group.*

Parameter	Control	S1				S2				
		50	300	2000	Sd	50	300	500	1000	Sd
LYM%	41.8	34.4	42.4	33.0	4-5	40.2	44.0	83.0	69.5	3-4
POLY%	57.6	64.6	56.8	66.7	3-5	59.0	55.4	17.0	30.0	1-3
PLT (10^9/L)	460.6	665.2	730.8	719.3	156-228	454.0	679.6	495.5	814.0	42-152
MCHC (g/dL)	34.4	33.5	33.8	34.0	0.3-1.0	34.8	33.9	33.2	32.1	0.1-4
MCH (pg)	21.9	21.4	21.5	21.9	0.6-1.5	20.9	21.3	19.0	18.4	0.5-0.8
MCV (fl)	63.7	63.6	63.6	64.7	2-4	60.5	62.1	61.6	58.7	0.3-3
Hct%	41.8	43.3	40.3	41.8	0.6-4	41.0	41.8	46.5	48.0	0.5-4
HGB (g/dL)	14.4	14.7	13.7	14.2	0.1-1	14.1	14.1	14.3	15.2	0.1-1
RBC (10^{12}/L)	6.6	6.8	6.4	6.5	0.1-0.5	6.7	6.6	7.5	7.9	0.2-0.5
WBC (10^9/L)	11.2	14.2	12.6	13.0	2-4	11.0	12.6	12.5	16.5	1-3

4. Discussion

The purpose of a water safety evaluation is to determine whether water treated with a new chemical is safe for humans to consume or not. DBNPA and BAC are two main biocides which are used for water treatment industry. BAC is a cationic surfactant used as a bactericide or preservative which inhibit bacteria and fungi growth. DBNPA is also applied as an antimicrobial agent in difgferent industries. The results show that the DBNPA is approximately twice as toxic as BAC in equal concentration and DBNPA is more toxic than BAC [3, 6].

The no-observed-effect-level (NOEL) obtained for BAC and DBNPA from dose-response curves was 1000 mg, and 500 mg / kg body weight, respectively. Acceptable daily intake (ADI) represents the amount of chemical residues that can safely be consumed per day over a human's lifetime without adverse effects. According to the formula, safe concentration was obtained 175 and 75.5 mg/kg, for BAC and DBNPA, respectively for 4 lit water consumption per day.

Biocides are widely used and the risk of accidental exposure is high, and as a result, there is a need to evaluate health problems. Biocides are used to control harmful and unwanted organisms and microorganisms. However, they are not only kill pathogens, but also they are kill non-pathogens, meaning they are dangerous to humans. In general, biocide exposure may occur as a result of ingestion, inhalation or skin contact. Poisoning is one of the most important health effects of chemicals, with more than 80,000 chemicals registered for use today. In order to control most of the chemicals that cause poisoning, more knowledge is needed about the mechanisms that cause side effects [3, 7, 8]. The findings of the present study demonstrated that the amount of TG in the blood of laboratory animals treated with BAC and DBNP was significantly changed compared to the control group. This biomarker was reduced in BAC group but it was raised in high doses of DBNP group. It should be noted that BAC and DBNP had no adverse effect on other biochemical parameters such as cholesterol, LDL, ALT, AST, ALP, BILI, CPK and LDH. Following consumption of chemicals, they eventually enters into the blood and they inevitably affect blood and hematological parameters. Hence, the blood parameters of the tested animals were considered. In this regard, 500 and 1000 mg of DBNP boosted lymphocyte count in exposed rats but the remaining hematological parameters displayed no statistically significant alteration

compared to the control group. The change in lymphocyte count observed in this group may be due to nutritional deficiency or stress due to weight loss [9]. In a recent study, Hye-Yeon Choi et al demonstrated that BAC induced significant changes in hematological parameters [10].

5. Conclusions

The results of our study showed that benzalkonium chloride and bromo nitrile propionamide cannot affect the blood and biochemical parameters, vital organs and important enzymes of the body. Finally, for two chemicals benzalkonium chloride and dibromo nitrile propionamide, the values of LD50 were 2346 and 1062 ppm, respectively. According to the same calculations, the amount of MRL for the two substances was calculated to be 175 and 87.50 ppm, respectively. Therefore, their use in various industries with the specifications mentioned is safe for human health.

Ethical Considerations

Compliance with Ethical Guidelines

All experiments were performed according to the ethical principles of working with laboratory animals, which was approved by the Faculty of Veterinary Medicine, University of Tehran (approval No of 7506023.6.17).

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Authors' Contributions

All authors made substantial and equal contributions to the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declared no conflict of interest.

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