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# 28-day inhalation toxicity of 3-methoxybutyl chloroformate in rats

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ABSTRACT

The 28-day repeated inhalation study was applied for hazard assessment of 3-methoxybutyl chloroformate (3-MBCF) in Sprague Dawley rats. Groups of five rats per sex were exposed 6 h/day, 5 days per week for 4 weeks to test substance concentration (ranging from 3 to 12 ppm) using a whole-body exposure system. At the terminal sacrifice, following blood collection and gross pathological examination, organ weights were determined and fixed organs were examined. The micronucleus test was performed using bone marrow cells. Exposure of 3-MBCF induced mortality at concentrations above 6 ppm. Decreases in body weight and food intake, hematologic alterations, organ weight changes, and gross and microscopic findings were seen even at the lowest concentrations of 3 ppm. Histopathology revealed principal test substance exposure correlated with lesions in the respiratory tract in both male and female rats above 3 ppm. Groups of male rats exposed above 6 ppm show microscopic lesions in spleens, livers, testes and epididymides; however, the micronucleated polychromatic erythrocytes frequency in bone marrow cells was not changed. Based on histopathology of the respiratory tract and other organs, the no observed adverse effect level (NOAEL) of 3-MBCF in the present study was less than 3 ppm.

## 1. Introduction

South Korea is one of the biggest chemical-producing countries in the world, and the 3rd largest producer in Asia after China and Japan [1]. In addition to producing large amounts of chemicals, hazardous chemicals are often found in workplaces, and the number of such chemicals has been steadily increasing. In South Korea more than 300 chemicals are newly registered each year, and there are presently approximately 43,500 types of chemicals used in Korean workplaces [2].

Recently, EU regulation no.1907/2006 on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) is the main basis for the environmental hazard assessment of industrial chemicals in each country [3]. In South Korea, The Ministry of Environment (MOE) reinforced the Registration, Evaluation, Authorization and restriction of Chemicals (K-REACH) to manage and inform the matters concerning the registration of chemical substances, as well as the review and assessment of the toxicity, hazards, and risks of chemical substances and products containing hazardous chemical substances [4]. MOE published a draft list of 518 existing chemical substances for registration under the Act on the Registration and Evaluation of Chemicals [5]. According to this list, manufacturers and importers who administer new chemicals or chemicals exceeding 1 ton per year should register it within 3 years after the publication date. The amount of ecotoxicological data requested depends on the production or import tonnage: higher tonnages require the provision of more extensive datasets, such as acute and chronic tests with fish and aquatic invertebrates, or reproduction studies.

3-Methoxy butyl chloroformate (3-MBCF; CAS No. 75032-87-0; Synonymes: 3-Methoxybutyl chloroformate; 3-methoxybutyl carbonochloridate; EINECS 278-058-3; AC1MI6SD) is a clear to light-yellow in color, water insoluble, and possesses a severe, pungent odor. 3-MBCF is used as a reactive chemical intermediate, especially for any chemical compound containing carbonate, pyrocarbonate, carbamate, urethane, and others, and may be used in organic chemical and plastics manufacturing. (PubChem Compound Database, 2005). 3-MBCF is a harmful material (Globally Harmonized System of Classification and Labelling

*Abbreviations:* ANOVA, analysis of variance; CT, computed tomography; EDTA, ethylenediamine tetraacetic acid; GHS, Globally Harmonized System of Classification and Labelling of Chemicals; GLP, Good Laboratory Practice; HCT, hematocrit; HGB, hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MNPCE, micronucleated polychromatic erythrocytes; MOE, The Ministry of Environment; NCE, normochromatic erythrocytes; NOAEL, no observed adverse effect level; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; PLT, platelets; RBC, red blood cell counts; RDW, red cell distribution width; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; SD, Sprague-Dawley; SPF, specific-pathogen-free; WBC, white blood cell counts; 3-MBCF, 3-methoxy butyl chloroformate

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of Chemicals (GHS) Acute toxicity, oral category 4) and causes skin irritation, serious eye irritation, and respiratory irritation (GHS skin corrosion/irritation-category 2; GHS serious eye damage/eye irritation-category 2A; GHS specific target organ toxicity, single exposure; respiratory tract irritation-category 3) (ECHA, 2017)

3-MBCF is included in the 518 existing chemical substances registered for their hazard to humans, animals, and the environment according to K-REACH [5], but there is little information associated with the biological hazard of 3-methoxybutyl chloroformate (3-MBCF). Only a single study reported about the pulmonary effects, including tracheolaryngeal inflammatory edema, bronchial dilation, and alveolar rupture using computed tomography (CT) in laboratory animals [6]. Here we confirmed potential toxicities of 3-MCBF in Sprague-Dawley (SD) rats exposed for 28 days. The study was performed in compliance with the Good Laboratory Practice (GLP) guidelines and the Organization for Economic Cooperation and Development (OECD) [7,8].

## 2. Materials and methods

### 2.1. Animals

Male and female specific-pathogen-free (SPF) SD rats aged 6–7 weeks were purchased (Orient Bio Inc., South Korea) and acclimatized for 11 days in polycarbonate cage with SPF conditions before the grouping. Exposures were conducted in inhalation chambers (Model No. SIS-20RG, Sibata Co., Japan) with individual wire mesh cages, three for each concentration of 3-MBCF plus a control chamber with HEPA filtered clean air under SPF laboratory conditions. The ambient temperature and relative humidity of the chamber was 22 ± 3 °C and 50 ± 20%, respectively, with a 12:12 h light:dark cycle (lights on at 8:00 am) with 150 ~ 300 lx of illumination. The rats received rodent chow (LabDiet 5002, Purina Mills., St. Louis, MO, USA) and tap water ad libitum. All animal protocols described in this study were approved by the Committee on Animal Research Committee of the Occupational Safety and Health Research Institute.

### 2.2. Chemicals and inhalation exposure

3-Methoxybutyl chloroformate (99.5% pure, CAS N0.:75032-87-0) was purchased from Sekiatofina Co., LTD. (South Korea, Lot No.3066), and the inhalation facilities are previously described [6] and will be summarized here briefly. The Environmental conditions were monitored every 30 min using equipment (Model No. ICS-21RG, Sibata Co. Ltd., Japan). The concentration-analysis of vaporized 3-MCBF (using a gas generator, Model No. VG-4R, Shbata Co. Ltd., Japan) in the chambers was performed every 15 min during exposure using gas chromatography (Model No. GCS-14BFS, SHIMADZU, Japan) with a flame-ionization detector and a column (15% DC-200; mesh 80/100, AW-DMCS, USA). The maximum concentration of 12 ppm and intermediate exposure concentrations of 3-MBCF were selected based on responses from preliminary studies.

### 2.3. Experimental design

Twenty rats of each sex were randomly assigned to four groups (n = 5); control (filtered air), 3, 6, and 12 ppm and were exposed to 3-MBCF for 6 h/day, 5 days/week for four weeks. Body weight data were collected twice a week for the first 2 weeks and then at least once per week after. Individual food consumptions were also collected once a week for 4 weeks. Clinical observations were recorded twice a day during exposure periods. At the end of the experiment all animals fasted for 12 h and were then anesthetized with pentobarbital (30 mg/kg, JW pharmaceutical, South Korea). Blood samples were then collected from the abdominal aorta. Then animals were sacrificed by exsanguination from the abdominal aorta and necropsy, including gross findings and organ weight determinations, was performed on all animals.

### 2.4. Hematology

Hematologic examination was performed using a hematology analyzer (CL-7200, Shimazu, Japan). Whole blood samples were collected in sample tubes containing ethylenediamine tetraacetic acid (EDTA2 K) and examined for the following parameters: white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and platelets (PLT).

### 2.5. Urinalysis

Three days before the necropsy, the urine samples were collected and analyzed using Uriscan 10 SGL strip and Uriscan S-300 (Youngdong Pharmatics, South Korea). The parameters including blood, bilirubin, urobilinogen, ketones, protein, nitrite, glucose, pH, specific gravity and leucocytes were measured.

### 2.6. Histopathological assessment

At the necropsy of all animals contained found dead animals, organs including the thymus, heart, testes, ovaries, lung with trachea, kidneys, spleen, liver, pancreas, stomach, and brain were removed and weighed. All organs/tissues were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedding. Tissue slides with 4 μm thickness prepared and stained with hematoxylin and eosin. Histological examination and capture of digital images was performed under the light microscope (Axioscope2, Zeiss, Germany).

### 2.7. Bone marrow micronucleus test

Bone marrow cells from the right femur of the rats were flushed using fetal bovine serum (FBS, Hyclone, GE Healthcare, IL, USA). The cell suspensions were centrifuged at 1000 rpm for 5 min, and the pellets were re-suspended and smeared on slides. After air-drying, the slides were fixed in methanol and stained with acridine orange solution (40 μg/mL). According to Hayashi et al. [9], the stained slides of bone marrow were observed for micronuclei, and the ratio of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) was counted. The frequency of micronucleated polychromatic erythrocytes (MNPCE) was scored in each 2000 PCE per animal using fluorescence microscope (Nikon, Optiphot-2, Tokyo, Japan).

### 2.8. Statistical analysis

Data were expressed as the mean ± standard deviation. The SPSS statistical system (Version 10.0; SPSS Inc., IL, USA, USA) was used to analyze parameters including body weight, food consumption, organ weights, hematologic data, and micronucleus assay data, followed by testing for variance homogeneity. Data were analyzed using the one-way analysis of variance (ANOVA) and Dunnett's multiple test or Kruskal-Wallis test with Bonferroni correct for comparison between the control and tested groups (except 12 ppm group). The incidence and severity for histopathologic findings were compared by uses of Fisher's exact probability test and Kruskal-Wallis test with Bonferroni correction. A value of $P < 0.05$ is considered as statistically significant.

## 3. Results

### 3.1. Environmental state and inhalation study

Data regarding the actual 3-MBCF concentrations and other conditions monitored in the exposure chambers are presented in Table 1. Briefly, the mean ( ± SD) concentrations measured during study were 3.3 ( ± 0.24), 6.1 ( ± 0.88), and 12.0 ( ± 1.48) ppm for nominal