



## Comparative Study on the EC<sub>50</sub> Value in Single and Mixtures of Dimethylformamide, Methyl Ethyl Ketone, and Toluene

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The aim of this research was to improve our understanding of human toxicity due to exposure to DMF, MEK, or TOL individually as compared to exposure to DMF-MEK or DMF-TOL mixtures, by comparing EC<sub>50</sub> values as well as the morphological changes in HepG2 cells treated with these substances. We found that there was marked cell necrosis in the groups treated with mixtures than in those treated with the compounds alone, and that the amount of cell death and the EC<sub>50</sub> value were more dependent on MEK and TOL than on DMF. Moreover, analysis of the changes in effective concentration curves revealed that MEK had an antagonistic effect on the human toxicity of DMF, whereas TOL had a synergistic effect. Accordingly, these results suggest that in workplaces involved in the manufacture of synthetic leather, mixtures of DMF and TOL should be avoided as much as possible in order to minimize environmental toxicity and protect the health of the workers.

**Key words:** Dimethylformamide, Methyl ethyl ketone, Toluene, Mixtures, EC<sub>50</sub>

### INTRODUCTION

Developments in chemical industry have led to a rapid increase in the development and production of chemical substances. As a result, the number of chemical substances being used in industry worldwide is over 100,000 (1), and the number being used in Korea alone is reported to be around 45,000 (2). While lifestyle convenience is enhanced by this increase in the number of chemicals, the fact that they are newly developed according to need and an increase in their usage has led to an adverse effect on people's health and on the environment. The 1984 chemical gas disaster in Bhopal, India, that claimed the died of thousands and caused injuries or disabilities to tens of thousands more; the 1986 Chernobyl nuclear power plant disaster in the former Soviet Union; and the mercury poisoning in Minamata Prefecture, Japan, are representative examples of environmen-

tal pollution. In Korea as well, there have been several major incidents that became a public issue, including exposure to carbon disulfide gas at the Wonjin Rayon factory; polyneuropathies caused by exposure to n-hexane, which was used as a cleaning agent in the electronics manufacturing industry; disorders of the reproductive organs caused by 2-bromopropane; and fulminant hepatic necrosis caused by exposure to dimethylformamide (CAS 68-12-2, DMF) produced by the synthetic leather manufacturing industry. Harmful chemical substances each possess their own particular physicochemical characteristics, which are responsible for the large differences in the extent of harm they cause to the human body, and so the accepted levels of exposure recommended for each substance are different (3). Researchers carry out experiments on animal and cells to investigate newly developed chemicals or those existing chemicals that have not yet been evaluated for human toxicity or their effect on the environment. Of these experiments, those that routinely use a laboratory-based approach to investigate the toxicity of chemical substances consider values such as the half effective concentration (EC<sub>50</sub>), half inhibition concentration (IC<sub>50</sub>), half lethal concentration (LC<sub>50</sub>), and half lethal dose (LD<sub>50</sub>) (4).

EC<sub>50</sub> and IC<sub>50</sub> evaluation is carried out at the cellular level, whereas LC<sub>50</sub> and LD<sub>50</sub> evaluation requires the use of experimental animals. Nevertheless, the majority of the

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study only tests single substances, and there is a lack of research regarding mixtures. There were limitations on performing the research on mixtures that the experimental design is more complicated than single substances, and that it required more expensive and time consuming (5). Nevertheless, there is much greater exposure to mixtures than to the compounds alone, and they exert a larger effect on workers in industry, who handle harmful chemical substances directly, as well as on natural ecosystems (6). Hence, we feel that there is an urgent need for more research into the human and environmental toxicity of mixtures.

When researching mixtures, it is important to determine how the physicochemical characteristics of the individual substances are changed through mutual interactions, and whether those changes have antagonistic or synergistic effects on the expression of toxicity. The results of such research can be used to help protect the environment and the health of workers, by preventing the mixing of substances that cause an increase in toxicity or, if that proves difficult, by reducing the mixing ratio and minimizing exposure (7,8).

In this study, we chose to investigate DMF, a known cause of fulminant hepatic necrosis in workers in the synthetic leather manufacturing industry as well as methyl ethyl ketone (CAS 78-93-3, MEK) and toluene (CAS 108-88-3, TOL), which are used in mixture with DMF. We analyzed and compared the effects of single substances and mixtures on cell morphology as well as the  $EC_{50}$  values.

## MATERIALS AND METHODS

**Materials.** The DMF, MEK, and TOL used in this experiment were of analytical grade (purity > 99.9%), purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell line used for cell cultures was human hepatocyte (HepG2), purchased from the Korean Cell Line Bank, and all other reagents were purchased from Sigma-Aldrich at analytical grade.

**Experimental determinations of physicochemical properties.** For studying the physicochemical properties of DMF, MEK, and TOL alone, we used the material safety data sheets (MSDS) from the Korea Occupational Safety and Health Agency and information provided by Sigma-Aldrich. For the mixtures, boiling point (9), specific gravity (10), rapid vapor pressure (rVP) (11), flash point (12), and lower explosive limit (13) were measured according to the Korean and American standard experimental methods.

**Cell culture and experimental determination of  $EC_{50}$ .** Ratios for the mixtures were determined on the basis of the ratios used in the synthetic leather manufacturing industry, and are presented in Table 1. Cell cultures were maintained in an incubator at 5%  $CO_2$ , 37°C, in DMEM (10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin) and MEM media. Cells were seeded at a concentration of  $5 \times 10^4$  cells/

**Table 1.** Classification of experimental groups and mixing ratio of chemicals

Groups	Mixing ratio (Vol./Vol.)
<i>Single</i>	
DMF	1.0
MEK	1.0
TOL	1.0
<i>Mixtures</i>	
DM1	DMF + MEK = 1.0 : 0.5
DM2	DMF + MEK = 1.0 : 1.0
DM3	DMF + MEK = 0.5 : 1.0
DT1	DMF + TOL = 1.0 : 0.5
DT2	DMF + TOL = 1.1 : 1.0
DT3	DMF + TOL = 0.5 : 1.0
DMT	DMF + MEK + TOL = 1.0 : 1.0 : 1.0

DMF, dimethylformamide; MEK, methyl ethyl ketone; TOL, toluene.

well on a 96 well plate (Corning, New York, USA) and were treated with chemical substances 24 hr later according to the groups in Table 1. After removal of the culture medium, a CCK-8 assay kit (Woongbee, Seoul, Korea) was used, dissolved to a concentration of 1/10 in DMEM. Cells were maintained in an incubator at 37°C for 1 hr 30 min after treatment, and the production of formazan was measured by the light absorption at a wavelength of 450 nm to determine the  $EC_{50}$ . Twenty-four hours after culturing, morphological changes in the HepG2 cells were observed using a TOMORO AcquCam (OLYMPUS, Japan) attached to an Olympus CKX41 microscope (OLYMPUS, Japan).

**Statistical analysis.** All experimental results were analyzed using SPSS 19 (SPSS Inc., Chicago, USA). For the comparison of physicochemical properties between single substances and mixtures, a one-way analysis of variance (ANOVA) was performed, and results are presented as mean ± standard deviation.

## RESULTS

**Experimental determination of physicochemical properties.** We measured the boiling point, specific gravity, rVP, flash point, and lower explosive limit for each experimental group and recorded the results in Table 2. These physicochemical properties showed a significant difference between the single substances and the mixtures ( $p = 0.0001$ ). In the case of DMF, as a single substance the boiling point was 153.0°C but, although there were slight differences depending on the mixture ratio, the addition of MEK (range: 93.8~114.4°C) or TOL (range: 118.4~127.9°C) significantly lowered the boiling point. The specific gravity and the flash point showed a similar result with boiling point. However, the rVP, which was 0.93 kPa for DMF alone, was dramatically increased by the addition of MEK

**Table 2.** Physicochemical properties and EC<sub>50</sub> value

Groups	CAS No.	Physicochemical properties					EC <sub>50</sub> (HepG2 cell) (mg/100 µl)
		BP (°C)	SG (g/ml)	rVP (kPa)	FP (°C)	LEL (%)	
<i>Single substance</i>							
DMF	68-12-2	153.0	0.948	0.93 ± 0.15	58.0	2.2	1.785
MEK	78-93-3	80.0	0.805	21.63 ± 0.15	3.0	1.8	3.068
TOL	108-88-3	110.0	0.864	6.87 ± 0.21	4.0	1.1	0.396
<i>Mixtures</i>							
DM1	-	114.4 ± 0.44	0.9042 ± 0.0001	8.40 ± 0.100	12.29 ± 0.298	2.627 ± 0.040	2.887
DM2	-	102.9 ± 0.07	0.8813 ± 0.0001	10.90 ± 0.173	6.14 ± 0.298	2.310 ± 0.046	2.765
DM3	-	93.8 ± 0.15	0.8565 ± 0.0006	14.57 ± 0.115	1.64 ± 0.271	2.027 ± 0.025	2.686
DT1	-	127.9 ± 0.10	0.9247 ± 0.0004	4.07 ± 0.058	21.09 ± 0.012	2.033 ± 0.042	0.837
DT2	-	123.7 ± 0.35	0.9129 ± 0.0002	4.87 ± 0.058	18.07 ± 0.465	1.733 ± 0.015	0.854
DT3	-	118.4 ± 0.32	0.8979 ± 0.0004	5.80 ± 0.100	13.32 ± 0.321	1.470 ± 0.010	0.873
DMT	-	103.5 ± 0.10	0.8780 ± 0.0003	10.43 ± 0.153	6.02 ± 0.515	1.810 ± 0.017	1.882
<i>p</i> value		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

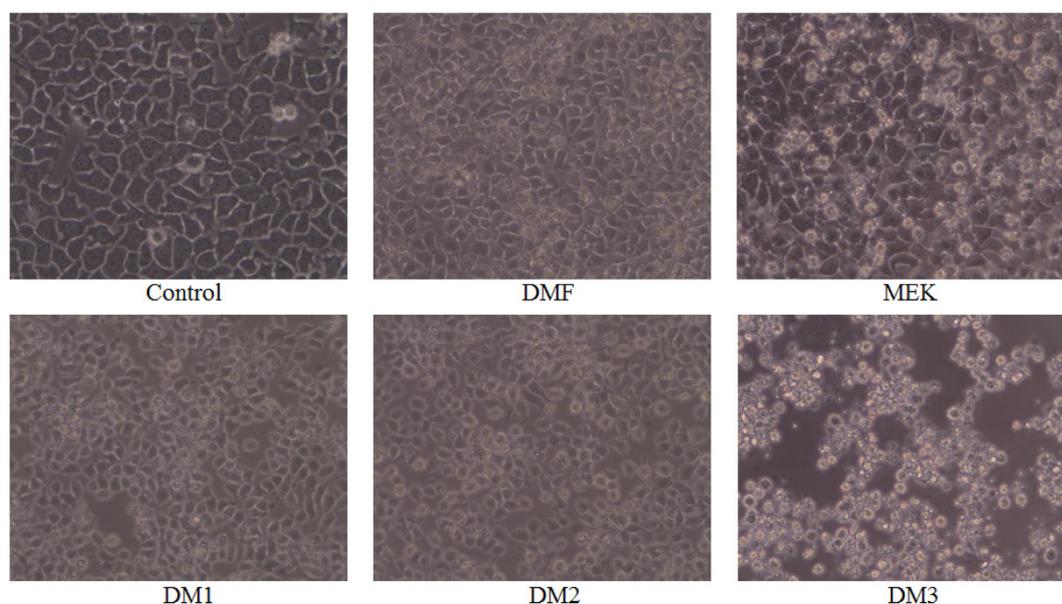
HepG2, human hepatocytes; BP, boiling point; SG, specific gravity; rVP, Rapid vapor pressure; FP, flash point; LEL, low explosion limit; DMF, dimethylformide; MEK, methyl ethyl ketone; TOL, toluene; DM1, DMF + MEK = 1.0 : 0.5; DM2, DMF + MEK = 1.0 : 1.0; DM3, DMF + MEK = 0.5 : 1.0; DT1, DMF + TOL = 1.0 : 0.5; DT2, DMF + TOL = 1.0 : 1.0; DT3, DMF + TOL = 0.5 : 1.0; DMT, DMF + MEK + TOL = 1.0 : 1.0 : 1.0.

(range: 8.40~14.57 kPa) or TOL (range: 4.07~5.80 kPa).

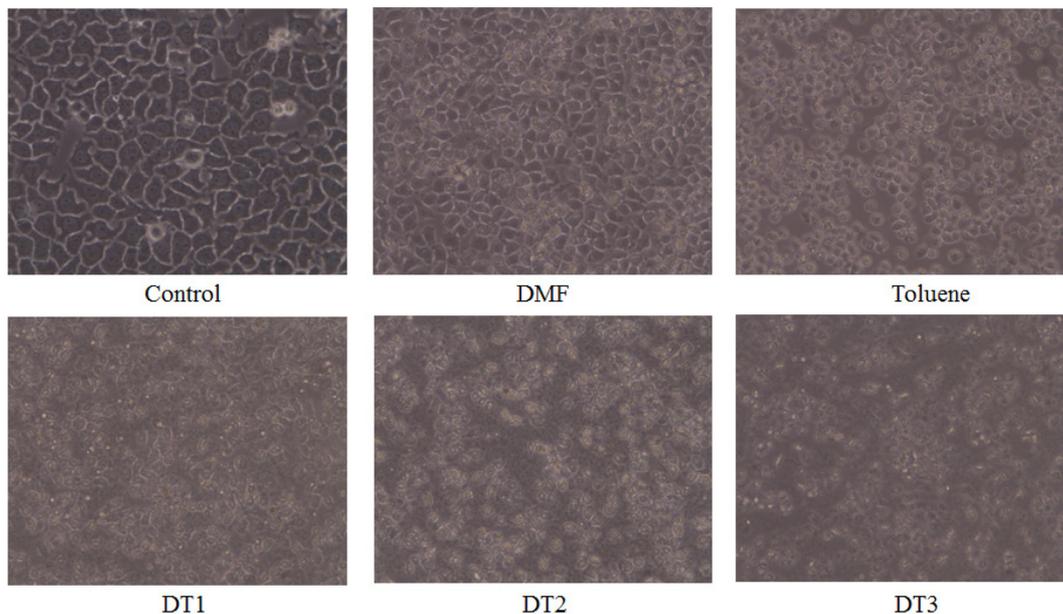
#### EC<sub>50</sub> value and morphological changes of HepG2 cells.

We observed the condition of HepG2 cells after treatment with the compounds alone or mixtures and presented the results in Figs. 1 and 2. In the groups treated with mixtures, there was greater cell necrosis than in the groups treated with either DMF, MEK, or TOL alone. In addition, we saw a difference in cell necrosis depending on the mixture ratio,

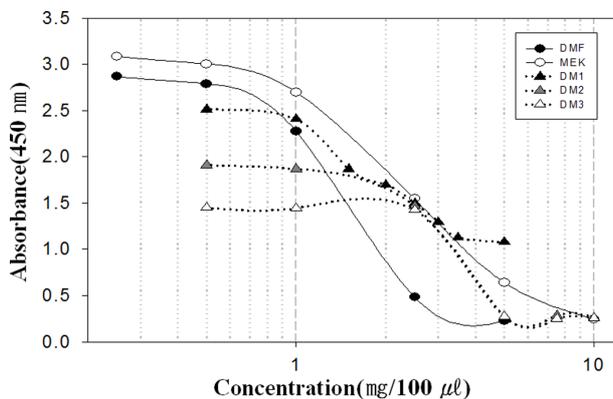
with DM3 (DMF : MEK ratio = 0.5 : 1.0) (Fig. 1) and DT3 (DMF : TOL ratio = 0.5 : 1.0) (Fig. 2) showing much greater cell necrosis than the other groups. Measurement of EC<sub>50</sub> values revealed TOL had the lowest EC<sub>50</sub> (DMF, MEK, and TOL: 1.785, 3.068, and 0.396 mg/100 µl. In the DMF-MEK mixture group, EC<sub>50</sub> was higher than for DMF alone (2.686~2.887 mg/100 µl), while that for the DMF-TOL mixture group was lower (0.837~0.873 mg/100 µl) (Table 2). On the basis of the changes in EC<sub>50</sub> and the concentra-



**Fig. 1.** Morphological changes of human hepatoma (HepG2) cells in untreated control and chemical-treated HepG2 cells. After treatment with 50 mg of chemicals for 24 hrs incubation, the HepG2 cells were directly examined under Olympus CKX41 (Olympus, Japan) and images of cell obtained using TOMORO AcquCam II Analyzer (Olympus Japan). Magnification, x20. DM1, DMF + MEK (mixing ratio = 1.0 : 0.5); DM2, DMF + MEK (mixing ratio = 1.0 : 1.0); DM3, DMF + MEK (mixing ratio = 0.5 : 1.0).



**Fig. 2.** Morphological changes of human hepatoma (HepG2) cells in untreated control and chemical-treated HepG2 cells. After treatment with 50 mg of chemicals for 24 hrs incubation, the HepG2 cells were directly examined under Olympus CKX41 (Olympus, Japan) and images of cell obtained using TOMORO AcqCam II Analyzer (Olympus Japan). Magnification, x20. DT1, DMF + Toluene (mixing ratio = 1.0 : 0.5); DT2, DMF + Toluene (mixing ratio = 1.0 : 1.0); DT3, DMF + TOL (mixing ratio = 0.5 : 1.0).

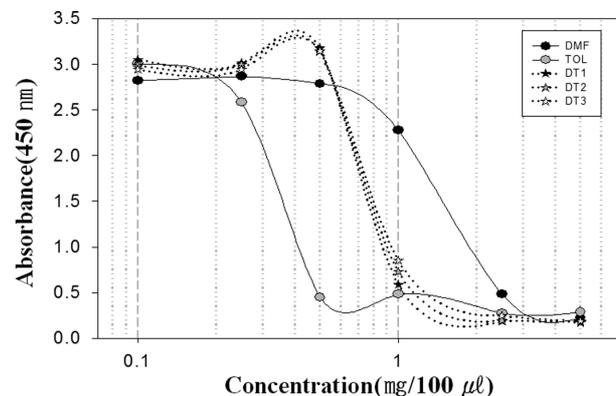


**Fig. 3.** Changes of  $EC_{50}$  value in HepG2 cells treated with DMF or MEK or both as described in Materials and Methods. DMF, dimethylformide; MEK, methyl ethyl ketone; DM1, DMF + MEK (mixing ratio = 1.0 : 0.5); DM2, DMF + MEK (mixing ratio = 1.0 : 1.0); DM3, DMF + MEK (mixing ratio = 0.5 : 1.0).

tions of single substances and mixtures, we found that the changes in  $EC_{50}$  in the DMF-MEK mixture and the DMF-TOL mixture were dependent on the presence of either MEK (Fig. 3) or TOL (Fig. 4).

## DISCUSSION

The aim of this study was to measure  $EC_{50}$  values and analyze morphological changes in HepG2 cells in order to better understand the ways in which toxicity to humans is



**Fig. 4.** Changes of  $EC_{50}$  value in HepG2 cells treated with DMF or TOL or both as described in Materials and Methods. DMF, dimethylformide; TOL, toluene; DT1, DMF + TOL (mixing ratio = 1.0 : 0.5); DT2, DMF + TOL (mixing ratio = 1.0 : 1.0); DT3, DMF + TOL (mixing ratio = 0.5 : 1.0).

affected by the form (single substance or mixture) in which chemical substances are handled.

We observed a much larger amount of cell necrosis in the groups treated with mixtures than in those treated with DMF, MEK, or TOL individually. We also found that the  $EC_{50}$  value was more dependent on MEK and TOL than on DMF.

Methods of analysis using biological monitoring on the basis of precise exposure levels of chemical substances (14) are being developed, and are consequently providing much

help in revealing mechanisms of human toxicity from chemicals and pathways of environmental toxicity. Metabolic intermediates formed by xenobiotic metabolizing enzymes are known, in most cases, to have a larger effect on human toxicity than the original chemical itself. DMF, which is widely used in the industry (15), is known to largely affect the liver (16), where the metabolic intermediate N-methylformamide (NMF) is produced during metabolism with the xenobiotic metabolizing enzyme cytochrome P-450(CYP)2E1 acting as catalyst. NMF is reported to have greater toxicity than DMF (15). The absorption pathways and metabolic processes involving MEK and TOL have been studied, and their target organs and the mechanisms of toxicity are well known (17,18).

As we mentioned in the introduction, DMF is sometimes used as a single substance in the synthetic leather manufacturing industry, but it is most commonly used in a mixture with either MEK or TOL. Consequently, research is underway on the metabolism of these mixtures, but the mechanisms of human toxicity of these mixtures are not clearly understood. Chang *et al.* (19) gathered urine samples from workers in the synthetic leather manufacturing industry, who had been exposed to mixtures of DMF, MEK, and TOL, and measured the concentration of NMF, a metabolic product of DMF. Mixtures with a higher concentration of MEK or TOL than DMF were shown to reduce the amount of NMF excreted, with the reported reason being that MEK and TOL competitively inhibit CYP2E1 activity. Kim *et al.* (8) showed that there was a significant difference in the boiling point, specific gravity, vapor pressure, and flash point of the mixtures than the individual substances and suggested that this may mean that the risk of exposure to DMF is higher for the mixtures. According to research concerning the cytotoxicity of benzene and benzene derivatives, a mixture of benzene derivatives and TOL is more harmful because of a reaction of the methyl side-chain of TOL (20). It has also been reported that cytotoxicity is related to lipophilicity (21). In the current experiment, we showed that when treating HepG2 cells with either DMF, MEK, or TOL individually or with DMF-MEK or DMF-TOL mixtures, the mixture groups had greater cytotoxicity than the individual substances. Based on our previous research (8), in which we showed that the octanol/water partition coefficient—related to lipophilicity—increases in the order DMF < MEK < TOL, we believe that the current results can be explained by an increase in lipophilicity when MEK or TOL are added to DMF. In addition, analysis of the change in the effective concentration curves suggests that MEK has an antagonistic effect on DMF toxicity, while TOL has a synergistic effect, though this is difficult to ascertain.

In conclusion, we suggest that preventing the use of DMF-TOL mixtures as much as possible in the synthetic leather manufacturing industry would help reduce environ-

mental toxicity and benefit the health of workers.

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