Toxicological Studies on Benzophenone-Type UV Filters: Environmental Exposure, Toxicokinetic, Genotoxic and Toxicogenomic Assessment

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Toxicological Studies on Benzophenone-Type UV Filters: Environmental Exposure, Toxicokinetic, Genotoxic and Toxicogenomic Assessment

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지금은 연구실을 떠나 있지만, 긴 시간이든 짧은 시간이든 독성학 실험실에서 인연을 맺게된 많은 분들게도 고마움을 전합니다. 인간미 넘치는 충고를 아끼지 않으시는 이행석 박사님, 멋진 선배이자 연구자로서의 열정을 보여주시는 서영록 박사님, 언니 같은 친근함으로 대해 주시는 최해연 박사님, 박사과정 입학동기이자 졸업동기인 염병수 예비 박사님, 잊지않고 축하해 주신 형태오빠와 효정이, 가끔식 전화를 통해서라도 언니를 격려해 주는 은영이, 정란이, 벌써 아기엄마가 된 혜정이, 따뜻하고 순수한 맘을 가진 영석이, 얼마전 까지 옆자리에서 조잘대던 귀염둥이 미순이, 모두들 너무나 소중한 인연입니다.

같은 실험실은 아니지만, 귀찮게 이것저것 물어보더라도 기꺼이 시간을 할애해 주신 표희수 박사님, 권오승 박사님, 가끔씩 복도에서 마주치더라도 웃음을 주었던 배수열 박사님, 동생이지만 언니 같은 넉넉함으로 힘들때에도 미소짓게 만드는 힘을 가진^^ 사랑하는 동생 혜정이, 택근이, 알고 지낸 시간을 짧지만 정많고 따뜻한 인성이, 이쁜이 애린이에게도 고마움을 전합니다.

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2007년 1월 …

한국과학기술연구원

독성학 실험실에서

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List of Abbreviations

AUC	Area under the curve
AUMC	Area under the moment curve
B[a]P	Benzo[a]pyrene
BH	Benzhydrol
BP	Benzophenone
BP-d_{10}	Benzophenone-d_{10}
$[B]^+$	Base peak ion
CDFBS	Charcoal dextran-treated fetal bovine serum
Cl	Total body clearance
C_{max}	Maximum plasma concentration
DHB	2,4-Dihydroxybenzophenone
DHMB	2,2’-Dihydroxy-4-methoxybenzophenone
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDCs	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GC-MS	Gas chromatography-mass spectrometry
HBP	4-Hydroxybenzophenone
HMB	2-Hydroxy-4-methoxybenzophenone
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IC_{20}	20% Inhibitory concentration
IDLs	Instrumental detection limits
IS	Internal standard
IWGTP	International Workshop on Genotoxicity Test Procedures
KFDA	Korea Food and Drug Administration
LD_{50}	50 % Lethal doses
LLE	Liquid-liquid extraction

LMPA	Low melting point agarose
MDLs	Method detection limits
MMS	Methyl methanesulfonate
MQLs	Method quantification limits
MRT	Mean residence time
MSD	Mass selective detector
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$[M]^+$	Molecular ion
NCT	National Center for Toxicogenomics
NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute for Occupational Safety and Health
NOES	National Occupational Exposure Survey
NTP	National Toxicology Program
OSHA	Occupational Safety and Health Administration
RME	Relative mean error
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
SD	Standard deviation
SIM	Selected ion monitoring
- S9	In the absence of S9 metabolic activation systems
+ S9	In the presence of S9 metabolic activation systems
$t_{1/2}$	Biological half-life
THB	2,3,4-Trihydroxylbenzophenone
T_{max}	Time to maximum concentration
V_z/F	Apparent volume of distribution

ABSTRACT

Toxicological Studies on Benzophenone-Type UV Filters: Environmental Exposure, Toxicokinetic, Genotoxic and Toxicogenomic Assessment

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UV filters of benzophenone (BP)-type are used primarily as photoinitiators, fragrance enhancers, as ultraviolet curing agents and occasionally, as flavor ingredients. They are also used in the manufacture of insecticides, agricultural chemicals and pharmaceuticals and as additives for plastics, coatings and adhesives. Especially, 2-hydroxy-4-methoxybenzophenone (HMB) is widely used as a UV stabilizer in skin moisturizing products and sunscreen lotions, usually in conjunction

with 2,2'-dihydroxy-4-methoxybenzophenone (DHMB). Therefore, this study investigated environmental exposure levels, metabolism, kinetic behavior, genotoxicity and specific-regulated genes of BP-type UV filters using overall toxicological approaches.

First, a novel method has been developed to determine and quantify seven organic UV filters simultaneously employing liquid (solid)-liquid extraction, derivatization with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and gas chromatography with mass spectrometry (GC-MS) in various environmental matrices. The UV filters determined were: BP, benzhydrol (BH), 4-hydroxybenzophenone (HBP), HMB, 2,4-dihydroxybenzophenone (DHB), DHMB and 2,3,4-trihydroxylbenzophenone (THB). Under optimal conditions, the high recovery (62-114 % and 60-125 % for water and soil, respectively) and the low relative standard deviation (RSD) values (less than 13.9% and 17.2% for water and soil, respectively) were indicated the high performance of this method. The detection limits of method were relatively low, ranging from 5 to 100 ng/L or kg and the quantification limits of method ranged between from 25 to 500 ng/L or kg for all test compounds. This validated method was applied to the analysis of seven BP-type UV filters in water and soil samples in Korea, collected between April and May 2003. The overall concentration of UV filters in the soil samples (500-18,380 ppt) was

higher than in water samples (27-204 ppt). Therefore the established method in this study was successfully applied to monitor the residue measurement of the BP-type UV filters in environmental water and soil samples.

Secondly, in rat blood, the simultaneous analysis of BP-type UV filters was optimized by GC-MS. The male Sprague-Dawley rats were used in this study and BP and HMB were administered orally at a dose of 100 mg kg^{-1} body weight. Plasma sample was adjusted to pH 9.5 and extracted with ethyl acetate for 20 mins. The organic solvent was reduced to dryness and the residues were derivatized with MSTFA and determined by GC-MS. Under optimal conditions, calibration curves showed a good linearity (r^2 > 0.999) and the recovery after extraction and concentration was above 76%. Intra-day and inter-day RSD values were within 9.87 and 13.89%, respectively, indicating good precision. The accuracy of the method expressed as relative mean error (RME) was below - 14.59% which was shown to be satisfactory. BP was mainly converted to BH and HBP in rat. Also, HMB was enzymatically converted to at least three intermediates. DHB was a major intermediate formed by *o*-demethylation, which in turn was converted to THB and DHMB by aromatic hydroxylation. The toxicokinetic parameters were presented by the noncompartmental analysis. The C_{max} value of BP was 2.06 ± 0.46 μg/ml and the T_{max} was 3.83 hr. The $t_{1/2}$ of BP was approximately 19.28 hr and the $AUC_{0-\infty}$ was

47.17 ㎍/ml·hr. The C_{max} value of HMB was 21.21 ± 11.61 μg/ml and the T_{max} was 2.71 hr. The $t_{1/2}$ of HMB was approximately 4.58 hr and the $AUC_{0-\infty}$ was 104.89 ㎍/ml·hr. The established method was successfully applied to various biological sample (urine and bile, etc.) for the determination of BP-type UV filters.

Thirdly, to evaluate the magnitude of DNA damage, the single cell gel electrophoresis assay was performed. The comet assay in alkaline condition is a rapid, sensitive, and reliable biochemical technique for evaluating single-stand DNA breakage in mammalian cells, as well as high throughput toxicity screening tool. From these results, BP and its metabolite, HBP, were observed statistically significant differences of tail moment values compared with negative control ($P < 0.05$). BH at tested all concentrations was not observed significant difference of tail moment. Also, HMB and its metabolite, DHB and THB, were revealed statistically significant differences of tail moment values compared with negative control ($P < 0.05$), except for DHMB. Therefore, we suggest that BP derivatives with hydroxyl group at the *para*-position such as HBP, DHB and THB possibly induce the single stranded DNA breakage in L5178Y cells.

Finally, these BP-type UV filters are thought to mimic estrogens in their action, and are called endocrine disrupting chemicals. In the previous studies, they have been seen to exert an uterotrophic effect *in vivo* and to stimulate cell proliferation of MCF-

7 breast cancer cells *in vitro*. Therefore, to identify genes elicited by three of BP-type UV filters, a human cDNA microarray analysis was carried out to MCF-7 cells, treated with BP, HBP and HMB using KISTCHIP-400 including 401 endocrine system related genes. Out of the analyzed genes, 32, 38 and 30 genes were identified showing significant changes (minimum 1.5-fold) in gene expression resulting from BP, HBP and HMB, respectively. Through the clustering analysis of gene expression profiles, 4 up-regulated and 6 down-regulated common genes by three UV filters were identified. The functions of these genes were found related to cell proliferation, thyroid hormone, regulation of transcription, metabolism and immune response. Among the genes, especially, 3 genes were induced and 3 genes were repressed by three BP-type UV filters as 17β-estradiol (E2). Therefore it is suggested that these genes may be associated with estrogenic effect of three UV filters. For the confirmation of the gene expression profiles identified from microarray analysis and the expression patterns of the other UV filters with estrogenic activity by dose- and time- dependent manner, real time RT-PCR will be performed. Taken together, this study provides evidence for previously unknown gene regulation by BP-type UV filters and E2, raising interesting possibilities regarding to the role of endocrine disruptors.

In summary, this thesis represents significant effects for exposure of BP-type UV filters viewed from different angles using toxicological tools. Through further investigation *in vitro* and *in vivo*, it should be characterized completely the toxic mechanisms of BP-type UV filters and applied as basic information for risk assessment and regulation establishment of BP-type UV filters.

Key Words : Benzophenone (BP), UV filters, Water, Soil, Gas chromatography-mass spectrometry (GC-MS), Toxicokinetics, Genotoxicity, Comet assay, Toxicogenomics, cDNA microarray

Chapter 1. Introduction to Benzophenone-Type UV Filters

1.1 Physical Properties and Use

Benzophenone (BP), a white crystal with a geranium- or rose-like odor, is an aryl ketone that is prepared by a Friedel-Crafts acylation using benzoyl chloride with an excess of benzene in the presence of anhydrous aluminum chloride (Furia and Bellanca, 1975; Kirk-Othmer, 1978; Merck Index, 1996). 2-Hydroxy-4-methoxybenzophenone (HMB), a plae cream-colored powder, occurs naturally in flower pigments and is synthesized for commercial use in various products (Stecher, 1958). BP is photochemically reactive and is incompatible with strong oxidizing and reducing agents; it may attack some plastics. Decomposition of BP produces toxic fumes of carbon monoxide and carbon dioxide (Sigma-Aldrich, 1988). Some of the physico-chemical properties of BP analogues are given in Table 1.1.

BP is used primarily as a photoinitiator and fragrance enhancer (Am. Paint Coatings J., 1990; Chem. Bus. Newsbase, 1991). BP is used in the manufacture of insecticides and agricultural chemicals and of hypnotics, antihistamines, and other pharmaceuticals; as an ultraviolet curing agent in sunglasses and ink; as an additive in

plastics, coatings, and adhesive formulations; and, occasionally, as a flavor ingredient. Production of benzophenone in the United States is estimated to range from 300,000 to 3,000,000 pounds per year (USEPA, 1991), with 100,000 pounds per year used in fragrances (Opdyke, 1973).

Especially, HMB is widely used as a UV stabilizer in cosmetic and plastic products. HMB is used in skin moisturizing products and sunscreen lotions, usually in conjunction with 2,2'-dihydroxy-4-methoxybenzophenone (DHMB) (Reynolds, 1982). In plastics manufacturing, HMB is used in surface coatings, and in the various polymers (Abramoff, 1978).

1.2 Environmental Exposure

BP-type UV filters have been reported to occur naturally in fruits and flowers (Furia and Bellanca, 1975). However, another source states that it does not occur naturally in the environment (Opdyke, 1973).

BP has been found in water samples taken from the Kitakysuhu area of Japan. In a 1989 study, collected tap water samples were analyzed using gas chromatography-mass spectrometry (GC-MS). BP was detected in tap water at a concentration of 8.8 ng/L (Akiyama et al., 1980). These state that domestic sewage

and industrial waste may represent the main sources of the contamination. Trace amounts of BP were detected in groundwater conducted to evaluate the effectiveness of rapid infiltration in removing organic compounds from waste water at a rapid infiltration site in Phoenix, Arizona. Although the sources of contamination were not reported, BP was found in the groundwater beneath the site at concentrations ranging from 0.009-0.045 mg/L (Tomson et al., 1981).

The relatively high estimated octanol/water partition coefficient and the water insolubility of BP indicate that it will partition into soil and sediment. BP is readily adsorbed to soil organic matter (USEPA, 1984). The adsorption of BP to soil is proportional to the organic content of the soil (OHMTADS, 1991). Although BP has been identified in the atmosphere, it would be difficult to determine whether its presence is due to its being a direct product of combustion or a secondary product of atmospheric degradation (Helmig et al., 1989). Leary et al. (1987) found that BP is a component of emissions from a standard residential oil burner. It has also been detected in surface and ground water samples, primarily from the discharge of untreated sewage and wastewater into waterways.

BP has been detected in the atmosphere of the Eggegebirge forest in Germany where, for the past decade, tree damage (particularly among spruce trees) has been observed. A total of 37 forest air samples were collected using sampling tubes

containing absorbent materials which were attached to personal sampling pumps. Three of the 37 samples were collected by passive sampling. Air samples were collected over periods of 1 to 24 hours. The samples were analyzed by an automatic thermodesorption device coupled with GC-MS. BP (concentration not specified) was one of 209 compounds identified. These stated that it was difficult to determine whether the presence of ketones, including BP, was the result of directly emitted substances (i.e., combustion products), or secondary products of atmospheric degradation (Helmig et al., 1989). BP was found to be a component of oil burner emissions in a 1987 study performed to characterize the oil burner emissions from a standard residential oil burner. Exhaust fumes from an oil burner, combusting No. 2 fuel oil, were collected after continuous and cyclic (5 minutes on, 10 minutes off) burning, and analyzed. BP was one of the principal compounds found in the chloroform extracts from the cyclic combustion samples. The source of this compound is not known. However, it is believed that it may result from oxidative pyrolysis of unreacted, or partially reacted, fuel in the post-flame regions of the oil burner combustion chamber (Leary et al., 1987). However, the US Environmental Protection Agency (EPA) reports that because of its low vapor pressure, BP is not expected to be present in significant quantities in the atmosphere (USEPA, 1984). BP may bioconcentrate (USEPA, 1984).

BP was one of 57 compounds positively identified from 35 air samples taken during the manufacture of rubber goods. The samples were obtained from 4 different locations: the vulcanization areas of a shoe sole factory and a tire retreading operation, and the extrusion areas of the tire retreading operation and an insulated cable manufacturer. Ambient air was collected on activated charcoal by means of personal samplers. Four sample tubes were collected at each location. BP was detected at levels of 0-1 mg/m^3 in the extrusion area of the electrical cable insulation plant using a GC-MS, it was not detected in the three other areas sampled. Although BP is not believed to be present in the raw materials, the authors speculate that it is produced, by an unknown mechanism, from the vulcanizing agent dicumyl peroxide which was used exclusively at the insulated cable manufacturing plant (Cocheo et al., 1983).

Based on the use of this compound as an additive in fragrances, toiletries, pharmaceuticals, insecticides, cosmetics, and flavor ingredients, consumer exposure may be significant. The concentration of BP in various consumer products is presented in Table 1.2.

Concentrations of BP in food products range from 0.57 ppm in non-alcoholic beverages to 3.27 ppm in frozen dairy products; it may also be an ingredient in baked goods, soft candy, gelatins, and puddings (NAS/NRC, 1979).

Data from the National Occupational Exposure Survey (NOES), conducted by the National Institute for Occupational Safety and Health (NIOSH) during the years 1981 to 1983, estimated that 41,520 workers, including 18,162 females, were potentially exposed to BP. These data were obtained from 1,809 companies. A classification of the NIOSH data is presented in Table 1.3.

1.3 Toxicity

The 50 % lethal doses (LD_{50}) in oral, intraperitoneal, and dermal administration of BP-type UV filters are given in Table 1.4. These data indicate that BP and its analogues are only slightly toxic. Groups of male rats (strain not specified) were fed diets containing 0.1 % or 1.0 % BP for 10 consecutive days. Feed consumption and body weights were slightly reduced in the 1.0 % group. Exposure concentration-dependent increases in absolute and relative liver weights and relative kidney weight were observed. Serum alanine aminotransferase activity of rats in the 1.0 % group was increased compared to that of the controls. Mild degenerative effects were observed in the liver and bone marrow of rats in the 1.0 % group, suggesting that the liver may be the primary target of the toxic effects of BP and that the bone marrow may also be targeted (USEPA, 1984).

BP was administered in feed to Sprague-Dawley rats at concentrations of 20 mg/kg body weight per day for 90 days or 100 or 500 mg/kg per day for 28 days (Burdock et al., 1991). Decreases in hematocrit values, erythrocyte counts, and hemoglobin concentrations were observed in females in the 100 and 500 mg/kg groups; a decrease in hemoglobin concentration was also evident in males in the 500 mg/kg group. Males in the 100 and 500 mg/kg groups had increased urea nitrogen concentrations; total bilirubin and protein were increased in males in the 500 mg/kg group and females in the 100 and 500 mg/kg groups. Males and females exposed to 100 or 500 mg/kg had increased albumin concentrations and absolute and relative liver and kidney weights. Histopathologic examination of the liver revealed hepatocellular enlargement with associated clumping of cytoplasmic basophilic material around the central vein in rats in the 100 and 500 mg/kg groups. Accordingly, the liver, kidney, and hematopoietic system were identified as targets of BP toxicity.

Slight skin irritation, evidenced by slight erythema and desquamation and slight to moderate edema, was observed in guinea pigs that received dermal applications of BP on the abdomen for 24 hours, under an occlusive wrap, or on the back, uncovered, for 10 days (USEPA, 1984). Additional exposures to BP failed to exacerbate the irritation, and no evidence of percutaneous absorption was reported. In a dermal study using the Draize method (Calas et al., 1977), BP was determined to

have medium irritation potential, with a primary cutaneous irritation index of 2.0 in rabbits. Additional experiments were conducted in guinea pigs to determine skin irritation and contact hypersensitivity induced by BP; in the open epicutaneous test, the Draize test, the maximization test, and a test with Freund's complete adjuvant, BP did not induce allergenicity in guinea pigs. Acetophenone, which is structurally related to BP, was tested for toxicity and tissue effects in rats fed diets containing up to 10,000 ppm for 17 weeks. No hematologic changes or gross or microscopic tissue changes were observed in exposed rats (IRIS, 1991).

1.4 Regulatory Status

The Occupational Safety and Health Administration (OSHA) has not established a permissible exposure limit for BP. The USEPA regulates process units that produce BP as an intermediate or a final product (40 CFR 60.489) (Office of the Federal Register, 1990a). The US Food and Drug Administration (US FDA) has approved the use of BP as a flavoring substance and adjuvant in accordance with the following conditions: It is used in the minimum quantity required to produce the intended effect, and otherwise in accordance with all the principles of good manufacturing practice. It is used alone or in combination with flavoring substances and adjuvants generally recognized as safe in food, prior-sanctioned for such use, or

regulated by an appropriate standard (21 CFR 172.515) (Office of the Federal Register, 1990b). The Council of Europe has approved the use of BP as an artificial flavoring substitute at 2 ppm (Opdyke, 1973).

American Conference of Governmental Industrial Hygienists has not recommended an exposure limit for BP. NIOSH has not recommended an exposure limit for BP. The flavor and extract manufacturers' association granted generally recognized as safe status to BP in 1965 (Opdyke, 1973).

In commercial products, the approved UV filters and their maximum allowed concentrations have been legislated by various regulatory authorities in Europe (EEC Directive, 1983), the USA (FDA, 1999) and Japan (SJA, 1985). The maximum authorized concentration of HMB in sunscreens is 10 % in Europe, 6 % in USA and 5 % in Japan. In Korea, according to the Korea Food and Drug Administration (KFDA) the maximum authorized level of HMB and DHMB is 5 and 3 %, respectively. Thus, the maximum content of such UV filters must be analyzed in order to check whether the products were prepared in accordance with official regulations, to know their environmental distributions and to assess by exposure levels in proportion to the level of discharge.

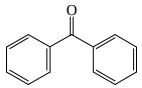
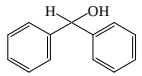
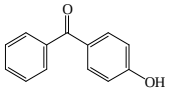
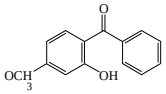
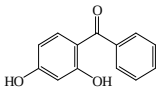
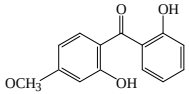
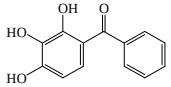
1.5 Objectives

This dissertation attempted to provide information concerning the environmental exposure, toxicokinetic, genotoxic and toxicogenomic assessment on overall toxicity of the BP-type UV filters.

The specific objectives were 1) to determine the exposure concentraton of BP-type UV filters in real water and soil samples, Korea, 2) to elucidate the metabolism and kinetic behaviour of BP-type UV filters in rats, 3) to identify the genotoxic effects of BP-type UV filters on DNA damage in the mouse lymphoma L5178Y cells, 4) to find the potential marker genes for estrogenic effect of BP-type UV filters and specific-regulated new biomarker genes by BP-type UV filters in the human breast cancer MCF7 cells.

Based on the above results, it has been identified that the potential risk of BP-type UV filters by overall toxicological studys in environment, animal, cell and gene levels. Therefore, this study provides basic information for the establishment of regulation for use of BP-type UV filters in environmental, food and other product.

Table 1.1. Structure and some physico-chemical properties of the test compounds

Compound	Chemical structure	Formula	Molecular mass	CAS number	b.p./ m.p. (℃)	Log K_{ow}[a]
Benzophenone (BP)		$C_{13}H_{10}O$	182.22	119-61-9	305 / 49	3.38
Benzhydrol (BH)		$C_{13}H_{12}O$	184.24	91-01-0	297-299 / 65-69	2.71
4-Hydroxybenzophenone (HBP)		$C_{13}H_{10}O_2$	198.22	1137-42-4	150-160 / 132-135	3.07
2-Hydroxy-4-methoxy benzophenone (HMB)		$C_{14}H_{12}O_3$	228.24	131-57-7	150-160 / 66	3.52
2,4-Dihydroxy benzophenone (DHB)		$C_{13}H_{10}O_3$	214.22	131-56-6	194 / 144-145	2.96
2,2'-Dihydroxy-4-methoxy benzophenone (DHMB)		$C_{14}H_{12}O_4$	244.24	131-53-3	170-175 / 68	3.82
2,3,4-Trihydroxy benzophenone (THB)		$C_{13}H_{10}O_4$	230.22	1143-72-2	- / 140-142	-

[a] K_{ow} = Octanol-water partition coefficient.

Table 1.2. Concentrations of benzophenone in consumer products

Product	Conc.	Ref.
Non-alcoholic beverages	0.50 ppm	Furia And Bellanca, 1975
Ice cream, ices, etc.	0.61 ppm	Furia And Bellanca, 1975
Candy	1.70 ppm	Furia And Bellanca, 1975
Baked goods	2.40 ppm	Furia And Bellanca, 1975
Soap	0.02-0.15%	Opdyke, 1973
Detergent	0.002-0.015%	Opdyke, 1973
Creams, lotions	0.004-0.015%	Opdyke, 1973
Perfume	0.09-0.3%	Opdyke, 1973

Table 1.3. National occupational exposure survey data for exposured worker to benzophenone

Description of Industry	Number of Company	Total Employees	Female Employees
Paper and Allied Products	181	1,581	---
Printing and Publishing	60	633	95
Chemicals and Allied Products	62	8,791	738
Leather and Leather Products	24	2,940	2,414
Stone, Clay, and Glass Products	17	3,220	2,717
Primary Metal Industries	47	2,752	---
Fabricated metal Products	346	4,145	1,024
Machinery, Except Electrical	117	6,530	5,230
Electrical and Electronic Equipment	118	550	320
Instruments and Related Products	43	1,495	120
Miscellaneous Manufacturing Industries	14	82	55
Trucking and Warehousing	61	607	303
Busness Services	24	893	187
Auto Repair, Services, and Garages	466	932	466
Health Services	228	6,366	4,493
TOTAL	1,809	41,516	18,162

NIOSH, 1991

Table 1.4. The LD_{50} values of benzophenone-type UV filters for oral, intraperitoneal, and dermal administration

Name	LD_{50}
BP	Rat (oral) : 1,900 mg/kg
	Mouse (oral) : 2,895 mg/kg
	Mouse (intraperitoneal) : 727 mg/kg
	Rabbit (dermal) : 3,535 mg/kg
BH	rat (oral) : 5,000 mg/kg
	rabbit (skin) : >5,000 mg/kg
HBP	mouse (oral) : 3,724 mg/kg
	rat (oral) : 12,086 mg/kg
HMB	Rat (oral) : > 12.8 g/kg
DHB	-
DHMB	-
THB	-

LD_{50} = 50 % lethal doses

USEPA, 1984

Chapter 2. Environmental Exposure Assessment of Benzophenone-Type UV Filters

2.1 Backgrounds

Currently, with the increased use of sunscreens, questions should be raised concerning the environmental impact of sunscreen ingredients. UV filters applied to the skin may contact with moisture when released from the skin during swimming or bathing (Thomas *et al*., 2004). In addition, indirect input (e.g., rubber off with towels, washed off during showering, etc.) through wastewater treatment plants is possible. Although the amounts of UV filters used are small compared to those of many other chemicals used everyday (e.g., soaps and detergents), the environmental consequences may still be significant, due to the possible direct input of surface waters. Thus, the maximum content of such UV filters must be analyzed in order to check whether the products were prepared in accordance with official regulations, to know their environmental distributions and to assess by exposure levels in proportion to the level of discharge.

Different instrumental techniques have been used to determine UV filters in sunscreen products. Organic UV filters have been determined by NMR spectroscopy

(Mori *et al*., 1996; Engelmann *et al*., 2001; Smernik *et al*., 2005), Raman spectroscopy (Cheng *et al*., 1997; Kolev *et al*., 2000; Kleimeyer *et al*., 2003), UV–Vis absorption spectroscopy (Chisvert *et al*., 2001), high-performance thin-layer chromatography (HPTLC) (Musial *et al*., 1997; Musial *et al*., 1998), high-performance liquid chromatography (HPLC) (Rastogi *et al*., 1998; Vanquerp *et al*., 1999; Scalia, 2000; Chisvert *et al*., 2001), GC (Hany *et al*., 1995; Felix *et al*., 1998) and especially GC-MS (Ro et al., 1994; Sakkas et al., 2003; Poiger et al., 2004; Giokas et al., 2004; Plagellat et al., 2006). GC-MS is the most common method and allows the accurate determination and confirmation of UV filters among many pollutants in environmental matrices. Since most of the BP-type UV filters have low sensitivity and volatility for GC, derivatization such as silylation has been used to overcome these drawbacks.

Studies on this topic are quite limited and have focused on the analysis of sunscreen agents in commercial formulation (Scalia, 2000; Chisvert *et al*., 2001). There are no extensive monitoring surveys on the occurrence and the exposure of UV filters. Although the method for water analysis employing liquid-liquid extraction (LLE) is rather obsolete, the originality of this study can be noted as new analysis methodology of BP-type UV filters in the environment. This work is novel in it that the determination of UV filters in soils has not been published before. Furthermore,

this study reports concentration in river water, a task which has also never been reported before.

Thus, this study investigated: (i) the development of a suitable analytical method to quantify the seven UV filters BP, benzhydrol (BH), 4-hydroxybenzophenone (HBP), HMB, 2,4-dihydroxybenzophenone (DHB), DHMB and 2,3,4-trihydroxybenzophenone (THB) in environmental samples by GC-MS and (ii) determination of the concentrations of these UV filters in real water and soil samples.

2.2 Materials and Methods

2.2.1 Reagents and Standard Solutions

BP was obtained from Sigma (St. Louis, MO, USA) and benzophenone-d_{10} (BP-d_{10}) as internal standard (IS) was supplied by Supelco (Bellefonte, PA, USA). BH, HBP, HMB, DHB, DHMB and THB were purchased from Aldrich (Milwaukee, WI, USA or St. Louis, MO, USA). Stock standard solutions were prepared in methanol containing 1000 mg/L of each compound. From these standards, working standard mixtures containing each compound at 0.1 and 1 mg/L were prepared daily in methanol, and used to spike the water and soil samples. A 10 mg/L working

internal standard solution was prepared with methanol. All standard and working solutions were stored in the dark at 4°C prior to use. The solvent used, acetone, ethyl acetate and methanol were of the highest available purity and were obtained from J. T. Baker (Phillipsburg, NJ, USA). Sodium chloride was obtained from Mallinckrodt (Mexico) and anhydrous sodium sulfate was supplied by J. T. Baker (Phillipsburg, NJ, USA). NaCl and Na_2SO_4 were baked in a furnace at 500°C for 8 h before use. The derivatization reagent, N-methyl-n-(trimethylsilyl) trifluoroacetamide (MSTFA) was obtained from Sigma (St. Louis, MO, USA). Pure water (18 mΩ) from Milli-Q water system apparatus (Milford, MA, USA) was used. All glassware were rinsed with purified water and HPLC grade solvents and dried before use.

2.2.2 Sample Collection

Water samples were collected from rivers (25 sites), lakes (6 sites) and industrial drainage (7 sites), in Korea (April 2003). Soil samples were collected from ground soil (33 sites) and sediment (15 sites), in Korea (from April to May 2003). The collecting locations for the river and lake are affected by Korea's four major water sources: Han, Gum, Youngsan, and Nakdong Rivers. These samples were selected from various areas in accordance with the city's municipal water and recreational water in which population density was also considered. Industrial drainage was collected from effluent (or outlet) of wastewater treatment plant from

various industrial areas. Partially identical locations were selected and from their sediments, sediment samples were collected from rivers and lakes. Ground soil was collected from various areas spread out in residential, park, commercial and industrial areas where population is dense. Non-contaminated blank soils were obtained in the vicinity of Korea Institute of Science and Technology, Seoul, Korea. The blank soils were washed with methanol, dried under room temperature and mixed in a porcelain dish prior to recovery test. Pure water was used for blank and recovery tests. The samples of water and soil were collected at nation-wide sites, and supplied by National Institute of Environmental Research, Korea. All sampling sites of water and soil are illustrated in Fig. 2.1. Upon arrival, the collected samples were immediately stored at 4^{o}C in the refrigerator until analysis.

2.2.3 Preparation of Water and Soil Samples

Determining trace concentrations of UV filters in natural waters and soil proved to be rather difficult due to background contamination in the laboratory. UV filters are ingredients in many cosmetics and personal care products such as sunscreens, soaps and shampoos. Due to their lipophilic nature, these compounds were easily transferred to glassware and consumables used during sampling and sample preparation. Thus, greatest care was taken to avoid sample contamination. Therefore, surgical gloves were worn during pre-cleaning of sampling containers,

sampling and sample preparation. Glassware was held separately and solvent was rinsed prior to use. Only previously unopened packages of solvents, chemicals and other supplies were used.

One hundred mL of water sample was added to a separatory funnel and 10 g of NaCl was added and dissolved by vigorous shaking. BP-d_{10} (10 μL of 10 mg/L solution in Methanol) was added as IS to reach the spike level of 1 μg/L. The sample was extracted with 50 mL ethyl acetate by liquid-liquid partitioning. The separatory funnel was agitated vigorously in a vertical shaker (D0647, Dongyang Inc., Seoul, Korea) for 20 min. After the separating of ethyl acetate layer by discarding an aqueous layer, 50 mL of 5 % NaCl solution was used to cleanse the organic layer. To remove residual moisture, the organic layer was combined with Na_2SO_4 in a beaker. Then the organic layer was transferred to a round flask and concentrated using an evaporator (Büchi 461, Switzerland) to approximately 3 mL. The extract was transferred to a centrifuge tube. The round flask was washed twice with small amount of ethyl acetate and washed solution from the flask was added to the centrifuge tube. The combined solution was evaporated to dry fully under reduced pressure and the residues were derivatized for analysis by GC-mass selective detector (MSD).

Ten grams of soil and sediment samples were added to a centrifuge tube and mingled homogeneously with the same amount of Na_2SO_4. BP-d_{10} as IS (10 μL of 10

mg/L solution in methanol) was added to reach the spike level of 10 μg/kg. The soil sample was extracted with 20 mL of methanol by vigorous shaking (Edmund Buchler 7400, Tubingen, Germany) for 20 min and centrifuged (RT 6000B, Sorvall Inc., New town, CT, USA) for 15 min at 1,660 x g. The methanol layer was transferred to a round flask and evaporated to the final volume of approximately 3 mL, which was transferred to a 15 mL centrifuge tube. 1 mL of 5 % NaCl solution was added to the centrifuge tube, and mixed in a vortex-mixer. After 5 mL of ethyl acetate was added, the sample was extracted by a shaker, centrifuged and put into a freezer (-30^{o}C) for the separation of the organic layer. The organic layer was transferred to a centrifuge tube and dried by evaporation under reduced pressure and the residues were derivatized for analysis by GC-MSD.

2.2.4 Derivatization

GC is widely used for UV filters analysis because of its inherent advantages such as simplicity, and sensitivity. Because of high polarity of UV filters, it is necessary to derivatize these analytes to increase the chromatographic efficiency. Many factors such as reaction time, derivatizing reagent and reaction temperature affect the entire derivatization. Therefore, the initial work involved optimizing the derivatization reaction and verifying complete derivatization. According to the optimized derivatization condition, the dry residues were treated with MSTFA (50

μL). After vigorous mixing, the solution was heated at 80°C for 30 min. Once the derivatization was completed, 1 μL of the derivatized extract was injected into the GC-MSD. Silylation using MSTFA increased the sensitivity and detection limits of all test compounds.

2.2.5 GC-MS Analysis

Determination of UV filters was performed on a GC-MSD (HP 6890 plus-HP 5973, Hewlett Packard, USA) and the software used was ChemStation (G1701AA, Version A.03.00, Hewlett Packard, USA). The samples were applied to the GC-MSD by an auto liquid sampler (HP 7673) for analysis.

For separation, a capillary GC column of 30 m length and 200 μm internal diameter with a 0.33 μm film thickness of stationary phase of Ultra 2 (5 %-diphenyl-95 % dimethylsiloxane) from Agilent Technologies was used. The flow rate of helium (Shinyang Oxygen Inc., Seoul, Korea) as carrier gas was 1 mL/min. The injector temperature was set to 280°C and sample injection (1 μL) was in splitless mode. The GC column temperature was programmed from 100°C, ramped at 6°C /min to 210°C, ramped at 25°C /min to 290°C, and held for 4 min. The interface was kept at 280°C and mass spectra were obtained at 70 eV. The standard samples were analyzed under full-scan mode and selected ion monitoring (SIM) mode conditions.

2.2.6 Quantification

To achieve enhanced selectivity and better detection limits, analyses were performed in SIM mode. Three representative ions were selected from the mass spectrum of each compound to identify and quantify the response under SIM mode. The characteristic fragment ions for the identification and quantification after silylation and retention time of UV filters are shown in Table 2.1. The quantitative analysis was performed by IS method using the peak area ratios relative to the BP-d_{10}.

2.3 Results

2.3.1 Separation of Benzophenone-Type UV Filters by GC-MS

The optimum experimental conditions for the quantification of seven UV filters were investigated by GC-MS system using the liquid (solid)-liquid extraction. The majority of the compounds are lipophilic and amenable to GC. Fig. 2.2 shows chromatograms obtained with the proposed GC-MS method under SIM conditions. It can be observed the good resolution of the peaks in run time lower than 23 min.

2.3.2 Mass Spectra of Benzophenone-Type UV filters before and after Silylation

The full-scan mass spectra of these compounds before and after silylation are shown in Fig. 2.3. THB was not detected without silylation because of the high polarity. After silylation, the tested UV filters achieved complete derivatization of all hydroxyl groups and showed remarkable increase in sensitivities (Ro *et al*., 1994). Therefore, the studied compounds were detected and quantified by monitoring the most abundant ion, called base peak ion ($[B]^+$) and the molecular ion ($[M]^+$) after derivatization. The silylated $[M]^+$ ion and TMS^+ ion (*m/z* 73) ion were obtained from all the silylated compounds. The most of $[B]^+$ of the UV filters showed strong $[M-C_6H_5]^+$ (BP, *m/z* 105; HBP, *m/z* 193), $[M-OSi(CH_3)_3]^+$ (BH, *m/z* 167) and $[M-CH_3]^+$ (HMB, *m/z* 285; DHB, *m/z* 343; DHMB, *m/z* 373; THB, *m/z* 431) ion peaks. So that, quantification ion of UV filters selected the $[M]^+$ ion or $[B]^+$ ion. Especially, of the seven UV filters, the *m/z* 182 ion of BP and *m/z* 270 ion of HBP were used with $[M]^+$ ion for the quantification. Although other ions ($[B]^+$ ion) can be used for the quantification, these ions gave better sensitivity than $[B]^+$ ions for determination and were selected because of the advantage of molecular weight. A second ion called the identification ion was also monitored for the purpose of confirming of the analyte. For calibration, the integrated peaks of quantification ion trace were used. In case of a positive result for a certain analyte, the ratio between quantification and identification ion had to match the respective ratio measured with an external standard beforehand.

Sometimes, the peak of UV filters in practical GC analysis overlap with those of other ingredients containing in environmental samples. In this case, the GC-MS spectrum information obtained from monitoring more than two identification ions is useful to confirm whether the particular UV filters are present or not. The quantification and identification ions are summarized in Table 2.1.

The use of IS of isotope-labeled compound of BP was essential for better reproducibility. BP-d_{10} should be used especially for the analysis of BP analogue-type UV filter rather than BP-d_5, because BP-d_5 has *m/z* 105 as $[B^+]$ ion, resulting in the formation of the same $[B^+]$ ion of *m/z* 105 in BP. BP-d_{10} has *m/z* 110 as $[B^+]$ ion and uses *m/z* 192 of $[M]^+$ ion for quantification.

2.3.3 Linearity of Calibration Curve

The linearity of the calibration curves is a measure of the range within which the results are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in a sample. For this analysis, the linear range of GC-MS for the determination of seven UV filters was tested by increasing amounts of standards at 10, 50, 100, 250, 500, 1000, 2500 ng/L and a fixed amount (1000 ng/L) of IS in purified water. The analytes were extracted and derivatized as described above (section 2.2.3 and 2.2.4). The experiment was repeated at standard

concentrations of 100, 500, 1000, 2500, 5000, 10000, 25000 ng/kg and a fixed amount (10000 ng/kg) of IS in purified soil. The ratio of the peak area of analyte ions to that of IS was calculated. The data agreed well with a linear fit for all analytes and the plot of residuals showed no obvious pattern. As seen in Table 2.2, all of the analytes exhibited good linearity and the concentration-peak area correlation coefficients (R^2) were greater than 0.996 except for HBP in soil and THB in water, for which the values were 0.983 and 0.977, respectively. The homogeneity of the variance was tested using the outcomes of the measurement of three independently prepared standard dilutions from the same stock solution of each concentration level. The variances of each of the three results for all concentrations were similar.

2.3.4 Limits of Detection and Quantification

The limits of detection was defined and determined as the minimum detectable concentration of analyte in spiked water and soil extract giving a signal-to-noise ratio (S/N) of 3. The limit of quantification is the lowest test compounds concentration that can be quantified in a sample with acceptable precision under the stated operational conditions of the method. It was determined as the analyte concentration corresponding to an S/N of 10. The instrumental detection limits (IDLs) were estimated from the direct injection of the standard mixture successively diluted until a concentration level corresponding to an S/N ratio of 3 was reached. The pure standard

solutions for IDLs were prepared in methanol and were analyzed after derivatization. As shown in Table 2.3, the IDLs varied between 1 and 5 pg. As shown in Table 2.4, the method detection limits (MDLs) were lower than 10 ng/L in water and 100 ng/kg in soil. Moreover, method quantification limits (MQLs) were lower than 50 ng/L and 500 ng/kg, in water and soil sample, respectively, depending on the extraction, evaporation and derivatization condition and the target compounds.

2.3.5 Accuracy and Precision

To further validate the accuracy of the proposed method, recovery testing was carried out by spiking a known amount of the standard to purified water and soil, which do not contain the test chemicals. The recovery was tested by evaluating the percentage of the seven UV filters extracted from spiked sample matrix at four concentration levels, 5, 10, 50 and 100 ng/ 100 mL, and 10, 25, 50 and 100 ng/ 10 g in five replicas for each water and soil matrix. The four spiked levels were chosen as they were considered representative of the level that could be found in water and soil in Korea and were above the estimated MDLs. As shown in Table 2.4, reasonable recoveries were observed for all tested samples. Recovery of the analytes from the each matrix appeared to be functions of the concentration and varied from 62 to 114 % in waters and from 60 to 125 % in soils. The relative standard deviation (RSD) values ranged from 2.4 to 13.9 % for water sample and from 2.1 to 17.2 % for soil

sample (Table 2.5). The high recovery and the low RSD values obtained from four spiked concentration levels indicate high performance and possible application of the method to real environmental samples.

Usally, the precision of the analytical method was represented as RSD. The intra-day precision was determined by analyzing five spiked water samples at 100 ng/L and soil samples at 1000 ng/kg within a given day. The RSD were calculated to be from 4.0 to 9.7 % and from 3.9 to 13.3 for water and soil samples, respectively. The inter-day precision was also determined by analyzing five replicates of water spiked at 100 ng/L and soil spiked 1,000 ng/kg on 5 different days to demonstrate procedure robustness. The RSD were found ranging from 6.7 to 11.8 % in water and from 4.3 to 14.7 % in soil. The method shows very good repeatability and reasonable reproducibility at such low concentration of analytes (Table 2.5).

2.3.6 Ruggedness and Stability

The ruggedness of the method was evaluated by carrying out the analysis using standard solution (100, 500 ng/L), same chromatographic system and the same column by different analysts. Small differences in area ratio and good constancy in retention times were observed from three different analysts. The RSD of less than 7.5 % for areas and 0.1 % for retention times were obtained. The comparable detector

responses obtained from different analysts indicated that the method is capable of producing results with high precision for different analysts.

Normally, standards and organic extracts from environmental samples such as water and soil were derivatized immediately before their chromatographic analysis and their temporal stability was not systematically studied. However, apparent degradation of the silylated BP-type UV filters was not detected after 1 months of storage at -20 ℃ in darkness. According to the previous studies, BP-type UV filters have been reported of their good thermal stability (Andrew *et al*., 1997) and photostability (Véronique *et al*., 1999).

The results therefore demonstrate that the UV filters studied can be simultaneously separated ad determined from water and soil samples by the proposed method, with good accuracy and precision.

2.3.7 Concentration of Benzophenone-Type UV filters in Water and Soil Sample

The developed method was applied to analyze the seven UV filters of BP-type collected from water samples of 38 sites (rivers, lakes and industrial drainages) and soil samples of 48 sites (ground soils and sediments) in Korea, between April and May 2003.

As shown in Table 2.6, the analytical results of UV filters were presented as concentration range, mean and frequency. In the case of water samples, UV filters were detected in rivers and lakes, the concentration for most of them were below the quantification limits. Instead, the industrial drainage showed relatively high concentration compared to rivers and lakes going over the quantification limit. Especially BH, a major metabolite of BP and a reduced form of its keto group was detected with the highest concentration of 204 ng/L. This shows that BP-type UV filters exist more in industrial drainages than in rivers and lakes. The major cause of water contamination of BP-type UV filters is affected more from industrial wastewater than from municipal or recreational water. Also, BH is an intermediate of pharmaceuticals, agrochemicals and other organic compounds and is thought to be more detected in industrial areas where it is widely used.

In the case of soil samples, seven UV filters were distributed evenly in the ground soil and sediment. The parent compound BP among these UV filters shows both high concentration (approximately 5,000 ng/kg) and frequency (above the 90%) in ground soil and sediment samples.

From the results of Table 2.6, the concentration of HMB and DHMB were identified at range from 730 to 3,880 and from 500 to 4,170 ng/kg, respectively in soil sample. HMB and DHMB were also quantified in the soil sample but came out under

the quantification limit in the water sample. This shows that even though the soil and water sample may not have the same contamination source, the BP-type UV filters exist more in soil than in water. Moreover, despite the fact that the initial contamination of the UV filters happens directly through water, the secondary contamination is thought to happen through the soil. Additionally, BP may be emitted by fuel combustion process and this is why it is detected in most soil samples (USEPA, 1984; Helmig *et al*., 1986; Leary *et al.*, 1987).

2.4 Discussion

Currently, the number of papers on UV filters is limited and there are no extensive monitoring surveys on the occurrence and exposure routes of UV filters, their ecological persistence or the kinds and amounts of these substances that have negative effects on people and other living organisms (Potera, 2000). Therefore, the exposure level of the UV filters in the environment must be monitored to assure the environmental distribution and to control these compounds.

Many studies worldwide confirmed the presence of several UV filters released into the aquatic environment through municipal or industrial activities. In a previous paper, BP has been detected in tap water at Kitakysuhu in Japan, and its main contamination source was derived from domestic sewage and industrial waste

(Akiyama *et al*., 1980). Trace amounts of BP were also found in the groundwater at Northern Croatia in Yugoslavia (Ahel, 1991) and in Netherlands (Zoeteman, 1981). As shown in Table 2.6, the results of these previous studies were similar to the concentration of our water samples.

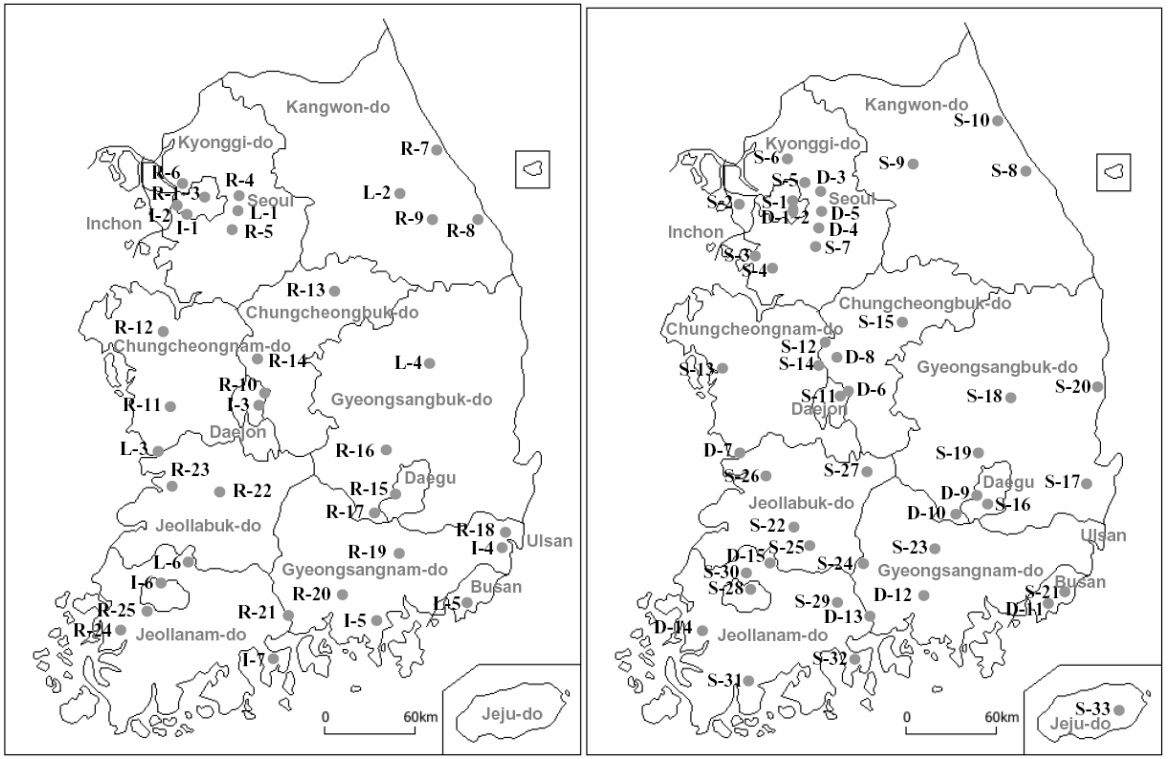
In addition, although BP was detected in Baltic Sea water (Ehrhardt *et al*., 1982) and water from Hamilton Harbour, Bermuda (Ehrhardt, 1987), UV filters could be difficult to determine in seawater sample, because of the increased dilution rate and high salt content. According to USEPA report (Bursey *et al*., 1982), BP has also been highly detected in wastewater effluents from the paint and ink industry, the pharmaceutical industry and the mechanical products industry.

In the case of soil, the sediment samples used in this experiment were collected from lakes and rivers sediments that are affected by municipal and recreational wastewater. When the results for the sediment and lake/water from the same location were compared, the UV filters for the lake and river appeared to be below the quantification limit while they were found high concentration in the sediment. This may have happened since the BP-type UV filers have high octanol/water partition coefficient and water insolubility hence most of them are accumulated in the sediment. According to the previous study regarding BP shows that our experiment results are

similar to the data reported in the Havel and Spree River sediments (Ricking *et al*., 2003).

Furthermore, HMB and DHMB are known as typical sunscreens. They are recommended for frequent application and reapplication after contact with water, and hence large amounts of sunscreen are used, without the consumers being aware of it. The US FDA has also approved HMB for its use as an indirect food additive (FDA, 1999). Therefore, HMB and DHMB are used in large quantities in various cosmetics and food-additives to protect human skin and food from ultraviolet radiation. These compounds may enter the natural environment such as river, lake, ground soil and sediment, directly or indirectly through water and waste treatment plants.

On the other hand, BP is mainly converted to BH, HBP, HBP-sulfate and DHB in rat hepatocytes (Nakagawa *et al*., 2000). HMB has been also shown to be metabolized into DHB, DHMB and THB (Nakagawa *et al*., 2002). Thus, if the parent compound, BP and HMB has been confirmed in the sample, it means that their metabolites actually exist in the environment. THB is a minor metabolite and was not detected during this experiment in both water and soil.

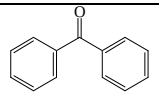
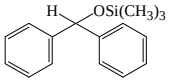
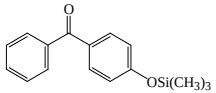
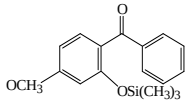
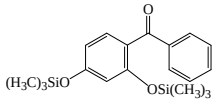
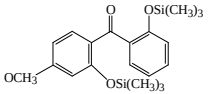
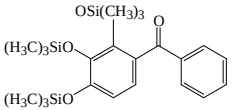


(a) Water (b) Soil

Figure 2.1. Sampling sites of water (a) and soil (b) in Korea.

R = river water, L = lake water, I = industrial drainage, S = ground soil, D = sediment.

Table 2.1. Selected ions (*m/z*) and retention time for quantitative analysis of UV filters after silylation

Compound	t_R (min)	Structure after silylation	Quantification ion (*m/z*)	Identification ions (*m/z*)
BP	14.39		182 $[M]^{+a}$	105 $[M\text{-}C_6H_5]^+$, $[B]^{+b}$, 77 $[C_6H_5]^+$
BH	14.55		167 $[M\text{-}OSi(CH_3)_3]^+$, $[B]^+$	256 $[M]^+$, 179 $[M\text{-}C_6H_5]^+$
HBP	20.37		270 $[M]^+$	255 $[M\text{-}CH_3]^+$, 193 $[M\text{-}C_6H_5]^+$, $[B]^+$
HMB	20.76		285 $[M\text{-}CH_3]^+$, $[B]^+$	300 $[M]^+$, 242 $[M\text{-}58]^+$
DHB	21.18		343 $[M\text{-}CH_3]^+$, $[B]^+$	358 $[M]^+$, 271 $[M\text{-}87]^+$
DHMB	21.73		373 $[M\text{-}CH_3]^+$, $[B]^+$	388 $[M]^+$, 299 $[M\text{-}OSi(CH_3)_3]^+$
THB	21.87		431 $[M\text{-}CH_3]^+$, $[B]^+$	446 $[M]^+$, 343 $[M\text{-}103]^+$

[a] $[M]^+$ = Molecular ion. [b] $[B]^+$ = Base peak ion.

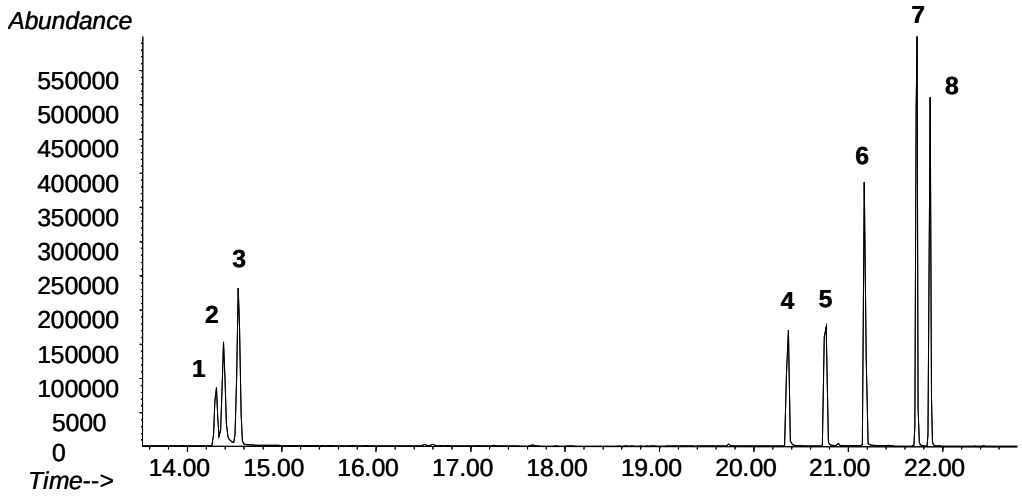


Figure 2.2. GC-MS total ion chromatogram of the benzophenone-type UV filters in selected ion monitoring mode.

Peak assignment: 1 = BP-d_{10}, 2 = BP, 3 = BH, 4 = HBP. 5 = HMB, 6 = DHB, 7 = DHMB, 8 = THB.

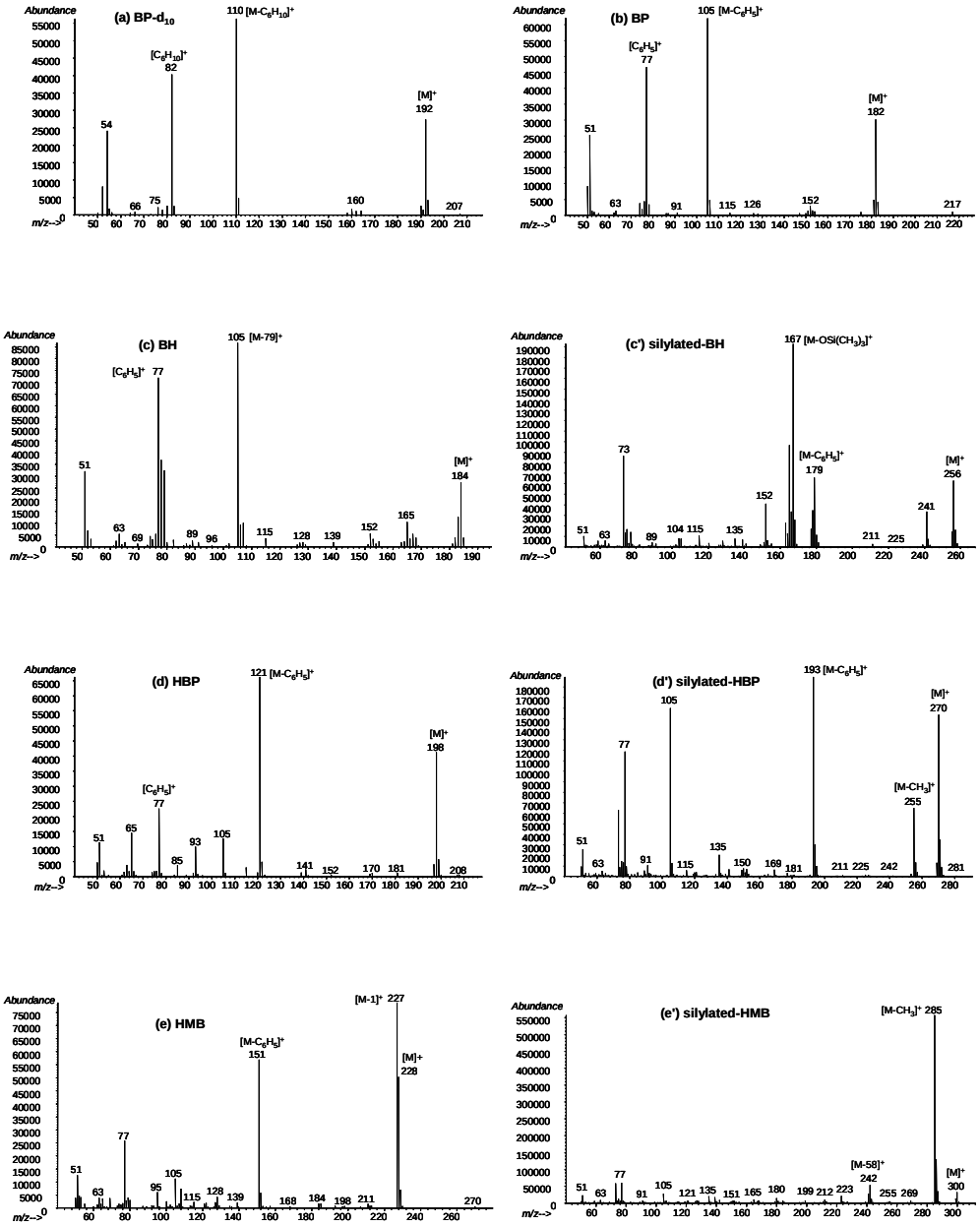
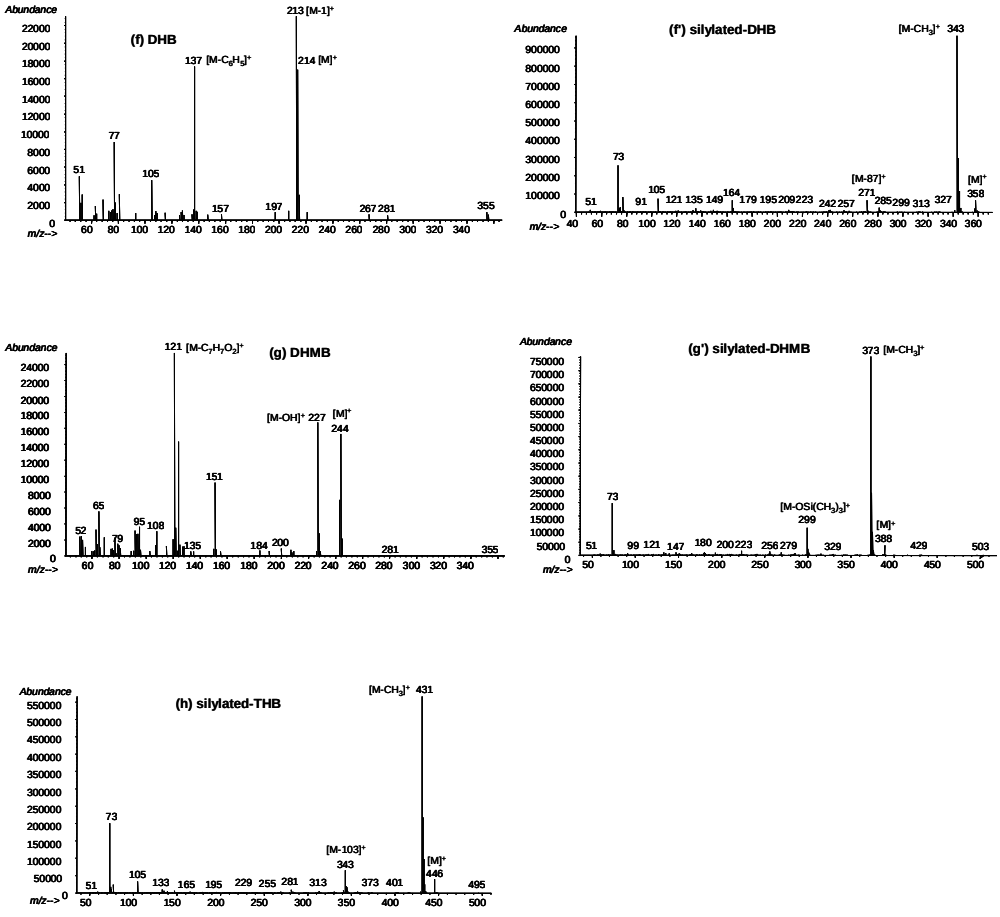


Figure 2.3. Mass spectra of internal standard and benzophenone-type UV filters before and after silylation.



(Figure 2.3. Continued.)

Table 2.2. Linearity for benzophenone-type UV filters in water and soil matrices

Compound	Matrix	Slope	Intercept	Correlation coefficients (R^2)
BP	Water	0.0128	0.0219	0.999
	Soil	0.0120	0.1593	0.998
BH	Water	0.0642	- 0.0807	0.999
	Soil	0.0436	0.2039	0.999
HBP	Water	0.0190	- 0.0287	0.998
	Soil	0.0262	0.0741	0.983
HMB	Water	0.0714	- 0.2270	0.996
	Soil	0.0594	0.1195	0.999
DHB	Water	0.0523	- 0.1971	0.997
	Soil	0.0950	- 0.1691	0.999
DHMB	Water	0.0634	- 0.2642	0.996
	Soil	0.0740	0.1658	0.999
THB	Water	0.0087	0.1060	0.977
	Soil	0.0109	0.1023	0.999

Three replicated measurements.

Concentration level = 1, 5, 10, 25, 50, 100 and 250 ng/ 100 mL water (or 10 g soil).

Table 2.3. Detection limits and quantification limits of benzophenone-type UV filters for the instrument and proposed method

Compound	IDLs[a] (S/N = 3)	MDLs[b] (S/N = 3)		MQLs[c] (S/N = 10)	
	(pg injected)	Water (ng/L)	Soil (ng/kg)	Water (ng/L)	Soil (μg/kg)
BP	2.5	5	100	25	500
BH	2.5	5	100	25	500
HBP	5	10	100	50	500
HMB	1	5	100	25	500
DHB	1	5	100	25	500
DHMB	1	5	100	25	500
THB	5	10	100	50	500

[a] IDLs = Instrumental detection limits.

[b] MDLs = Method detection limits.

[c] MQLs = Method quantification limits.

Dilution factor (D) = ($V_{initial}$ / V_{final}), D for water matrix: 100 mL / 50 μL = 2000, D for soil matrix: 10 g / 50 μL = 200.

Table 2.4. Recovery data for benzophenone-type UV filters in water and soil matrices

Spiked level (ng/L)	Values	Water (%) BP	BH	HBP	HMB	DHB	DHMB	THB	Spiked level (ng/kg)	Values	Soil (%) BP	BH	HBP	HMB	DHB	DHMB	THB
50	Mean	77	88	110	111	103	114	85	1000	Mean	115	111	99	75	82	78	88
	SD	10.4	2.9	9.2	11.1	14.4	10.3	10.7		SD	4.1	2.4	4.8	9.9	5.0	6.3	5.9
	RSD	13.5	3.3	8.3	10.0	13.9	9.0	12.6		RSD	3.5	2.1	4.9	13.3	6.1	8.1	6.7
100	Mean	85	97	91	108	106	99	112	2500	Mean	125	99	115	81	84	80	92
	SD	6.7	11.2	9.4	11.4	8.9	6.2	7.5		SD	7.8	3.6	5.2	7.2	8.2	6.6	6.6
	RSD	7.9	11.6	10.3	10.5	8.4	6.3	6.7		RSD	6.2	3.7	4.5	8.9	9.8	8.2	7.2
500	Mean	98	84	81	113	109	97	98	5000	Mean	102	76	88	71	66	68	81
	SD	2.4	4.8	3.3	8.4	6.8	6.6	8.3		SD	14.2	7.3	11.1	12.1	8.5	8.3	6.0
	RSD	2.4	5.7	4.1	7.4	6.3	6.8	8.5		RSD	14.0	9.7	12.7	17.2	12.7	12.1	7.4
1000	Mean	100	62	65	90	92	76	99	10000	Mean	105	107	73	77	62	60	89
	SD	3.6	5.7	6.2	9.8	9.6	8.9	12.5		SD	3.4	4.5	2.7	7.2	3.7	2.7	9.2
	RSD	3.6	9.2	9.6	10.8	10.4	11.8	12.7		RSD	3.2	4.2	3.6	9.4	6.0	4.5	10.3

Five replicated measurements.

Table 2.5. Precision of benzophenone-type UV filters for the instrument and proposed method

Compound	Intra-day[a] (RSD, %)		Inter-days[b] (RSD, %)	
	Water	Soil	Water	Soil
BP	5.8	3.9	6.7	4.3
BH	7.0	5.6	11.6	5.9
HBP	6.2	4.9	9.1	10.8
HMB	9.7	13.3	11.8	14.7
DHB	8.4	6.1	9.6	9.9
DHMB	6.3	8.1	7.7	11.5
THB	4.0	5.9	7.9	10.2

Concentration spiked =100 ng/L in water, 1000 ng/kg in soil.

[a]Intra-day = five replicated measurements.

[b]Inter-day = five replicated measurements, five different days.

Table 2.6. Concentration of benzophenone-type UV filters in water and soil samples

Compound	Values	Water (ng/L)		Soil (ng/kg)		
		River (25 sites)	Lake (6 sites)	Industrial drainage (7 sites)	Ground soil (33 sites)	Sediment (15 sites)
BP	Range	NQ[a]	ND[b]	32 ~ 51	820 ~ 16550	1520 ~ 9730
	mean	-	-	44	4550	4730
	Frequency	-	-	4 / 7 (57 %)	32 / 33 (97 %)	14 / 15 (93 %)
BH	Range	NQ	NQ	204	510 ~ 6950	530
	mean	-	-	204	1800	530
	Frequency	-	-	1 /7 (14 %)	13 / 33 (39 %)	1 / 15 (7 %)
HBP	Range	NQ	85	NQ	1060 ~ 4910	18380
	mean	-	85	-	3010	18380
	Frequency	-	1 / 6 (17 %)	-	3 / 33 (9 %)	1 / 15 (7 %)
HMB	Range	NQ	ND	27	730 ~ 3880	ND
	mean	-	-	27	2650	-
	Frequency	-	-	1 /7 (14 %)	5 / 33 (15 %)	-
DHB	Range	47	ND	NQ	NQ	ND
	mean	47	-	-	-	-
	Frequency	1 / 25 (4 %)	-	-	-	-
DHMB	Range	NQ	NQ	ND	500 ~ 4170	500 ~ 2140
	mean	-	-	-	1670	950
	Frequency	-	-	-	5 / 33 (15 %)	12 / 15 (80 %)
THB	Range	ND	ND	ND	ND	ND
	mean	-	-	-	-	-
	Frequency	-	-	-	-	-

[a] NQ = Not quantified (below the MQLs). [b] ND = Not detected.

Chapter 3. Toxicokinetic Assessment of Benzophenone-Type UV Filters

3.1 Backgrounds

The most important property of the BP-type UV filters is the ability to absorb and dissipate ultraviolet radiation. BP-type UV filters have been used to protect materials subject to discoloration or deterioration when exposed to sunlight. The widespread use of BP-type UV filters as photostabilizers in cosmetics has led to the exposure of million of consumers on a daily basis (Cosmetic Ingredient Review Panel, 1983). Exposing unprotected skin to ultraviolet light (primarily the UVB; 290-320 nm) over a long period of time promotes premature ageing of the skin and skin cancer. The BP-type UV filters have a broad absorption spectrum in the 200-350 nm range, and have been claimed to protect against both UVA and UVB radiation (Kaidbey *et al*., 1987). Also, BP-type UV filters have been used as sunscreen to ptotect patients using drugs from photosensitization, phototoxic or photoallergic reactions (FDA, 1978). HMB also known as oxybenzone, is a monomethoxylated and monohydroxylated derivative of the parent compound (Sahral *et al*., 1957). The National Toxicology Program (NTP) conduscted some oral and deramal toxicity studies on HMB in rats and mics. Body weight gain was

decreased at higher dietary levels. In both species, relative liver weight was found to be increased after oral exposure. There were also observations of decreased sperm concentration in the cauda epididymis in mice and rats exposured to 5 % HMB in the diet (NTP, 1991). However, these findings were unconfirmed by more recent studies. Although some aspects of the persistence and metabolism of BP and HMB following oral administration to rats have been reported in the scientific literature (El Dareer, 1986), the work of previous investigators lacks data on the rate of absorption and elimination from plasma, area under the plasma concentration-time curve. It is therefore the purpose of this study (i) to develop a suitable analytical method to quantify the BP-type UV filters in plasma samples by GC-MS and (ii) to determin the BP and HMB toxicokinetics in rats after oral administration.

3.2 Materials and Methods

3.2.1 Reagents

BP was obtained from Sigma (St. Louis, MO, USA) and BP-d_{10} as IS was supplied by Supelco (Bellefonte, PA, USA). BH, HBP, HMB, DHB, DHMB and THB were purchased from Aldrich (Milwaukee, WI, USA or St. Louis, MO, USA). Stock standard solutions were prepared in methanol containing 1000 mg/L of each

compound. From these standards, working standard mixtures containing each compound at 1, 10 and 100 mg/L were prepared daily in methanol, and used to spike the blood samples. A 10 mg/L working internal standard solution was prepared with Methanol. All standard and working solutions were stored in the dark at 4^{o}C prior to use. The solvent used, acetone, ethyl acetate, acetonitrile and methanol were of the highest available purity and were obtained from J. T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid and boric acid were obtained from Junsei Chemical Co. (Japan), and Na_2SO_4 was supplied by J. T. Baker (Phillipsburg, NJ, USA). Corn oil and MSTFA were obtained from Sigma (St. Louis, MO, USA). Entobar (Phentobarbital Sodium) was purchased from Hanlim Pharm. Co., LTD.

3.2.2 Animals Handling and Dosing

Male Sprague−Dawley rats (8-10weeks, 300±25 g) were purchased from Deahan Biolink Co., LTD (Korea). The animals (7 per chemical group) had free access to water and a standard diet (Han Lim Lab. Animal Co.) until 24hr prior to being used in experiments, at which time only food was removed, and kept under standard conditions (12 h day/night cycle, temperature 21−25 °C, humidity 45−55%).

Animals were administered orally BP and HMB (100 mg/kg body weight), dissolved in corn oil. To obtain a homogeneous dosing solution, BP and HMB were homogenized in corn oil during 5 min using an ultrasonicator (Branson 5510, Switzerland). A dosing volume of 4 ml/kg bw of corn oil was applied.

3.2.3 Collection and Preparation of Blood Sample

Animal was initially anesthetized with phentobarbital sodium (50mg/kg, i.p.). Blood samples were collected from the rat femoral artery into the heparinized polyethylene tube (PE-50, Daejong Instrument Industry Co., LTD) at predetermined time points up to 24hr after administration. The Blood samples were immediately prepared by centrifugation (15 min at 9000 rpm). Plasma samples were also stored at −70 °C.

Plasma sample (100 μl) was thawed at room temperature, added to an equal volume of acetonitrile in 1.5 mL Eppendorf tubes (100 μl), vortex mixed, and centrifuged at 15,000 rpm for 5 min to pellet precipitated proteins. Aliquots of the supernatant were treated with 6N HCl (200 μl) to hydrolyze bound compounds for 1hr in a heating block (100 ℃). After hydrolysis, samples were allowed to cool, adjusted pH 8.5 with borate buffer, and added the internal standard (10 ppm x 20 μl ; 200 ng) to each sample. For LLE of analyte and IS, 5 mL of ethyl acetate were

added and shacked for 20 min. A subsequent centrifugation for 10 min at 3000 rpm separated the organic phase from the plasma phase. Then the organic phase was evaporated to dryness. The residue was derivatized (30 min at 80℃) for analysis by GC- MSD.

3.2.4 GC-MS Analysis

Determination of UV filters was performed on a GC-MSD (HP 6890 plus-HP 5973, Hewlett Packard, USA) and the software used was ChemStation (G1701AA, Version A.03.00, Hewlett Packard, USA). The samples were applied by an auto liquid sampler (HP 7673) for analysis.

For separation, a capillary GC column of 30 m length and 200 μm internal diameter with a 0.33 μm film thickness of stationary phase of Ultra 2 (5 %-diphenyl-95 % dimethylsiloxane) from Agilent Technologies was used. The flow rate of helium (Shinyang Oxygen Inc., Seoul, Korea) as carrier gas was 1 mL/min. The injector temperature was set to 280^{o}C and sample injection (1 μL) was in splitless mode. The GC column temperature was programmed from 100^{o}C, ramped at 6^{o}C /min to 210^{o}C, ramped at 25^{o}C /min to 290^{o}C, and held for 4 min. The interface was kept at 280^{o}C and mass spectra were obtained at 70 eV.

Three representative fragment ions were selected from the mass spectrum of each compound to identify and quantify the response under SIM mode. The quantitative analysis was performed by IS method using the peak area ratios relative to the BP-d_{10}.

3.2.5 Toxicokinetic Analysis

Toxicokinetic calculations were performed on each individual set of data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by noncompartmental method (Gabrielsson *et al*., 1994). The following parameters were generated by the program: (i) biological half-life, ($t_{1/2}$), calculated from the slope of the terminal phase; (ii) area under the curve (AUC), where AUC was calculated to infinity according to the linear trapezoidal rule, $AUC = AUC_{0\text{-}t} + AUC_{t\text{-}inf}$.; (iii) area under the moment curve (AUMC), where AUMC was calculated to infinity according to the linear trapezoidal rule; (iv) maximum plasma concentration (C_{max}); (v) time to maximum concentration (T_{max}); (vi) total body clearance (Cl), where clearance = dose/AUC; (vii) apparent volume of distribution calculated based on the terminal phase(V_z/F); and (viii) mean residence time (MRT), calculated using AUMC/AUC.

3.3 Results

3.3.1 Separation of Benzophenone-Type UV Filters in Rat Blood by GC-MS

In the present study, we investigated a rapid specifie GC-MSD method under SIM conditions using the LLE for the analysis of seven BP-type UV filters in rat plasma in order to evaluate its toxicokinetics. The optimum experimental conditions for the quantification of seven UV filters could be observed the high sensitivity and good resolution of the peaks (no shown data).

3.3.2 Linearity of Calibration Curve

The linearity of the calibration curves is a measure of the range within which the results are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in a sample. For this analysis, the linear range of GC-MS for the determination of seven UV filters was tested by increasing amounts of standards at 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 an 100 mg/L and a fixed amount (2 mg/L) of IS in blank plasma. The analytes were extracted and derivatized as described above (section 3.2.4). The ratio of the peak area of analyte ions to that of IS was calculated. The data agreed well with a linear fit for all analytes and the plot of residuals showed no obvious pattern. As seen in Table 3.1,

all of the analytes exhibited good linearity and R^2 were greater than 0.9992 in rat plasma sample. The homogeneity of the variance was tested using the outcomes of the measurement of three independently prepared standard dilutions from the same stock solution of each concentration level. The variances of each of the three results for all concentrations were similar. Under the experimental conditions, the lower limit of detection was found to be approximately 0.01 μg/mL in rat plasma sample at a S/N of 3.

3.3.3 Intra-day and Inter-day Precision and Accuracy

The intra-day and inter-day vriabilities were determined by quantitating five replicates at concentrations of 0.5, 1 and 5 μg/mL using the GC-MSD method on the same day and five consecutive days, respectively. The RSD was calculated from the found concentrations as follows: precision (% RSD) = [standard deviation (SD) / Mean] x 100. The accuracy was calculated from the added concentration and the mean vlues of found concentration as follows: accuracy (% relative mean error (RME)) = [(Found – Added) / Added] x 100.

As shown in Table 3.2, the overall mean precision, defined by the RSD, ranged from 0.27 to 9.87 % in within a given day and from 0.96 to 13.89 % in five different days. Analytical accuracy, expressed as the percent difference mean found

values compared with known concentration varied from -14.59 to 3.81 % in within day and from -11.47 to 7.56 in different days. Thus, the intra-day and inter-day accuracy and precision were found to be acceptable for the analysis of plasma sample in support of toxicokinetic studies.

3.3.4 Recovery

To further validate the extraction efficiency of the proposed method, recovery testing was performed at three concentration levels, 0.5, 1 and 5 μg/mL in five replicate. Recoveries were measured by comparison of the peak area values of non-extracted standards versus extracted standards of spiked plasma at the same concentration. As shown in Table 3.3, reasonable recoveries were observed for all tested samples. Recovery of the test chemicals varied from 76 to 114 % in plasma. The RSD values were less than 10.34 %. The recoveries of seven BP-type UV filters fell well within the predefined limits of acceptability. Therefore, the method was suitable for the quantitation of test chemicals from toxicokinetic studies.

3.3.5 Toxicokinetics of Benzophenone-Type UV Filters

The validated method was used to analyze plasma sample from a toxicokinetic study in rats. The Toxicokinetic profile of BP-type UV filters was

investigated in male rats after oral administration of 100 mg/kg body weight of BP and HMB.

To detect and identify metabolites of BP and HMB, blood samples from BP- and HMB-treated rats were analyzed by GC-MSD. In blood samples collected before administration, peaks with parent compounds (BP and HMB) and its metabolites were not present or below the limit of detection. The separation of the blood sample from BP-treated rats gave three peaks. These results suggest the presence of two major metabolites (HBP and BH) (Fig. 3.1). Also, the separation of the blood sample from HMB-treated rats gave four peaks. These results suggest the presence of two major metabolites (DHB and DHMB). An additional metabolite (THB) with a high polarity was present in low concentrations (Fig. 3.2). Concentration of BH and THB in the plasma samples were at the limit of detecton in all samples analyzed and therefore can not quantified.

Blood samples collected at different time points after oral administraton were analyzed. Experimental results on plasma concentration-time course of parent compounds and its metabolites are given in Fig. 3.1 and 3.2. The toxicokinetic curves reflect the fact that the disposition of BP in rat plasma exhibited a peak concentration (C_{max}, 2.06 ± 0.46 μg/mL) after about 4 hr of BP administration (100 mg/kg), followed by a slow elimination phase. After a single oral dose of HMB, the

compound was absorbed rapidly. The C_{max} of 21.21 ± 11.61 µg/mL was attained after 3 hr (T_{max}) of administration.

Plasma concentration was measureable up to 24hr. The $t_{1/2}$ of BP and AUC were 19 hr and 47.17 ± 5.52 µg/mL·hr, respectively. The $t_{1/2}$ of HMB was 4.6 hr and AUC was 104.89 17 ± 23.82 µg/mL·hr. The V_z/F relates to the amounts of test compound in the body to the concentration of the test compound in the blood. Clearance of test compound occurs the amount of test compound eliminated per unit time depends on the amount (concentration) of the test compound in the body compartment. These toxicokinetic datas were shown in Table 3.4. These parameters are in agreement with those reported previously (Kadry *et al.*, 1995).

3.4 Discussion

The results represented clearly indicate rapid absorption of BP and HMB from the gastrointestinal tract. Structurally BP and HMB is a diphenyl ketone. The presence of two aromatic rings in its molecule confers some degree of lipophilicity to the compound, thus allowing faster absorption of the chemical from the gastrointestinal tract and faster disappearance (biphasic parrern) from plasma. The disappearance from plasma may be due to redistribution to compartments other than blood.

In the previous study, the tissue distribution of HMB was reported that the liver contained the highest amount of HMB, followed by the kidney. The study also showed that the main route of extraction of HMB during the 96 hr experimentation priod following oral adminitration was urine, followed by feces as a secondary route. Over 75 % of HMB excreted in urine occurred after 6-12 hr, while 90 % of the fecal excretion occurred in the first 24 hr following oral administration (Kadry *et al*., 1995).

The total amount of parent compound recovered in urine and feces after the 96 hr experimentation period was less than 60 % of the administered dose (Okereke *et al*., 1993), suggesting the formation of metabolites. The presence of these metabolites was noted by the detection of some peaks with retention times corresponding to standard peaks of seven UV filters during GC-MSD analysis.

The toxicokinetic parameters of BP and HMB was only determined in our experiment after acid hydrolysis. This is due to binding of BP and HMB to plasma protein. On considering the presence of a hydroxyl group and ketonic groups on the molecule and the possibility of hydrogen binding of these moieties to plasma macromolecules, strong protein binding has been demonstrated for many drugs and chemicals with similar functional groups in the molecule (Bos *et al*., 1988; Zini *et al*., 1988).

Table 3.1. Linearity of benzophenone-type UV filters in rat blood

Compound	Slope	Intercept	Correlation coefficients (R^2)
BP	0.1136	- 0.0212	0.9992
BH	0.4136	- 0.0091	0.9998
HBP	0.3359	- 0.0463	0.9998
HMB	0.8284	- 0.0323	0.9999
DHB	1.2052	0.1610	0.9994
DHMB	1.0228	0.1556	0.9992
THB	0.1374	0.0538	0.9993

Three replicated measurements.

Concentration level = 10, 50, 100, 250, 500, 1000, 2500, 5000 and 10000 ng/100 µL plasma.

Table 3.2. Intra-day and inter-day precision and accuracy of benzophenone-type UV filters for the GC-MS method

Compound	Intra-day				Inter-day			
	Concentration (μg/mL)		R.S.D.	RME	Concentration (μg/mL)		R.S.D.	RME
	Added	Found	(%)	(%)	Added	Found	(%)	(%)
BP	0.50	0.447	0.71	- 10.54	0.50	0.443	5.52	- 11.47
	1.00	0.991	1.69	- 0.91	1.00	0.999	0.96	- 0.06
	5.00	4.768	1.20	- 4.64	5.00	4.832	1.87	- 3.35
BH	0.50	0.436	3.25	- 12.84	0.50	0.465	3.48	- 7.05
	1.00	0.990	3.36	- 1.02	1.00	0.976	4.18	- 2.36
	5.00	4.842	3.05	- 3.17	5.00	5.378	3.48	7.56
HBP	0.50	0.450	3.58	- 9.98	0.50	0.472	2.16	- 5.66
	1.00	0.996	3.01	- 0.38	1.00	1.012	2.88	1.25
	5.00	5.042	3.43	0.85	5.00	4.785	4.05	- 4.31
HMB	0.50	0.486	5.38	- 2.75	0.50	0.506	1.97	1.17
	1.00	0.992	2.92	- 0.78	1.00	0.969	2.48	- 3.10
	5.00	5.047	5.30	0.93	5.00	4.823	4.61	- 3.54
DHB	0.50	0.427	4.02	- 14.59	0.50	0.448	1.84	- 10.39
	1.00	0.985	2.84	- 1.53	1.00	0.972	2.47	- 2.80
	5.00	4.967	2.97	- 0.66	5.00	4.776	5.42	- 4.48
DHMB	0.50	0.495	3.82	- 0.97	0.50	0.485	3.51	- 2.97
	1.00	1.000	3.17	0.01	1.00	0.941	3.62	- 5.94
	5.00	5.191	3.04	3.81	5.00	5.043	5.08	0.85
THB	0.50	0.431	3.47	- 13.87	0.50	0.448	13.89	- 10.44
	1.00	0.954	9.87	- 4.57	1.00	0.970	2.08	- 2.99
	5.00	4.701	0.27	- 5.98	5.00	5.117	4.53	2.35

RSD (relative standard deviation) = (SD / mean) x 100, RME (relative mean error) = [(Found-Added) / Added] x 100

Table 3.3. Recovery data for benzophenone-type UV filters in rat blood

Spiked Level (μg/mL)	Values (%)	BP	BH	HBP	HMB	DHB	DHMB	THB
0.5	mean	80.70	75.86	97.92	114.23	93.29	99.78	75.51
	S.D.	0.85	5.48	1.26	3.92	1.99	5.20	6.02
	R.S.D.	1.06	7.22	1.29	3.43	2.13	5.21	7.97
1	mean	98.90	90.51	107.55	111.40	102.75	111.63	100.56
	S.D.	0.84	12.95	1.89	3.32	1.33	1.69	7.00
	R.S.D.	0.84	14.31	1.75	2.98	1.29	1.51	6.97
5	mean	98.01	92.90	104.59	110.28	104.43	110.40	94.88
	S.D.	1.00	8.52	2.62	1.98	2.11	3.06	9.81
	R.S.D.	1.02	9.17	2.51	1.79	2.02	2.77	10.34

RSD (relative standard deviation) = (SD / mean) x 100

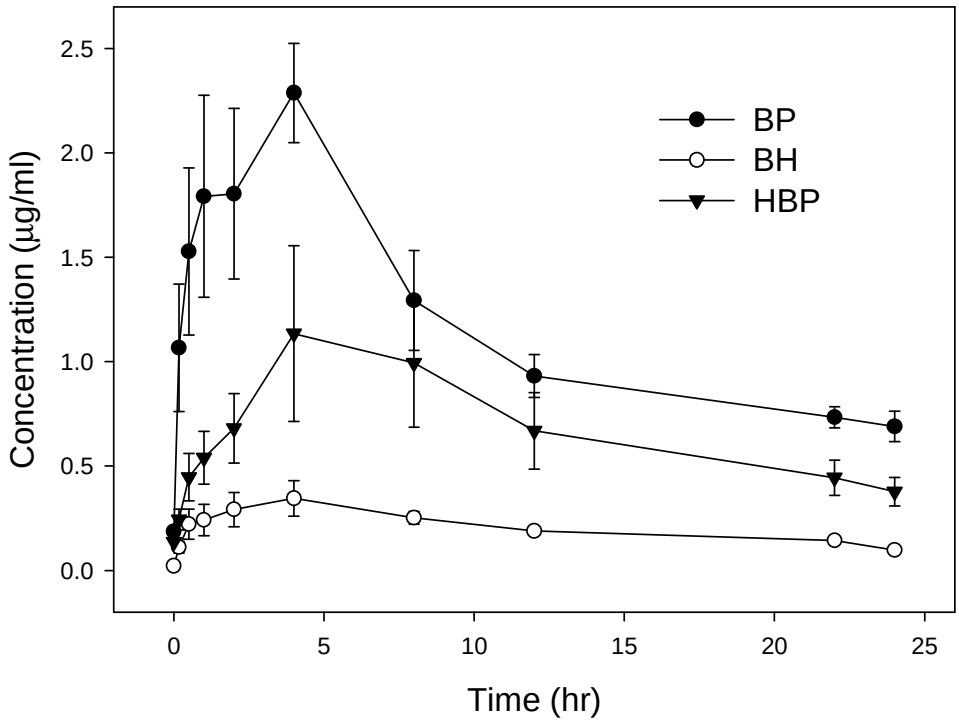


Figure 3.1. Plasma time courses of benzophenone and its metabolites after oral administration in male rats.

Administration dose = 100 mg/kg bw (n = 5).

BP = benzophenone, Two metabolites : HBP = 4-hydroxybenzophenone, BH = benzhydrol.

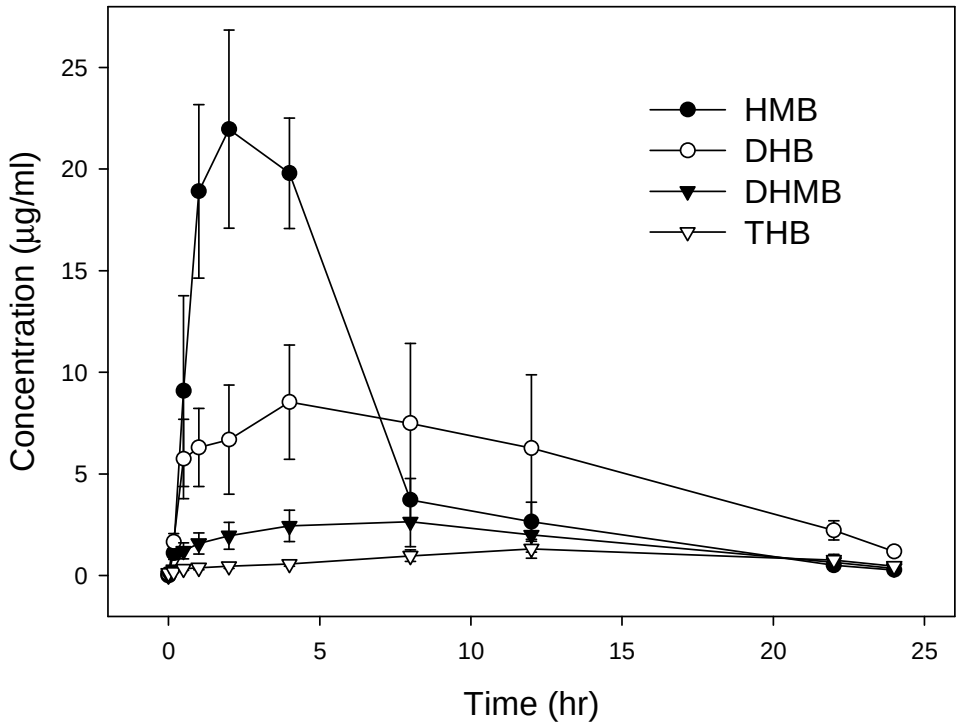


Figure 3.2. Plasma time courses of 2-hydroxy-4-methoxybenzophenone and its metabolites after oral administration in male rats.

Administration dose = 100 mg/kg bw (n = 7).

HMB = 2-hydroxy-4-methoxybenzophenone, Three metabolites : DHB = 2,4-dihydroxy benzophenone, DHMB = 2,2'-dihydroxy-4-methoxybenzophenone, THB = 2,3,4-trihydroxy benzophenone.

Table 3.4. Toxicokinetic parameters of benzophenone and 2-hydroxy-4-methoxybenzophenone after oral administration in rat

Parameters	BP	HMB
$AUC_{0\rightarrow\infty}$ [a] (μg/ml·hr)	47.17 ± 5.52	104.89 ± 23.82
T_{max} [b] (hr)	3.83 ± 2.14	2.71 ± 0.61
C_{max} [c] (μg/ml)	2.06 ± 0.46	21.21 ± 11.61
$T_{1/2}$ [d] (hr)	19.28 ± 8.54	4.58 ± 0.48
V_z/F [e] (ml/kg)	53.50 ± 16.43	9.79 ± 2.93
MRT[f] (hr)	27.40 ± 11.70	7.11 ± 0.80
Cl/F[g] (ml/hr/kg)	2.20 ± 0.22	1.33 ± 0.32

Values are expressed as mean ± SE (BP, n=5; HMB, n=7).

Animals were administered BP and HMB 100 mg/kg body weight by orally.

[a] area under the concentration-time curve from time zero to infinity; [b] time to maximum concentration; [c] maximum plasma concentration; [d] half life; [e] apparent volume of distribution; [f] mean residence time; [g] apparent clearance

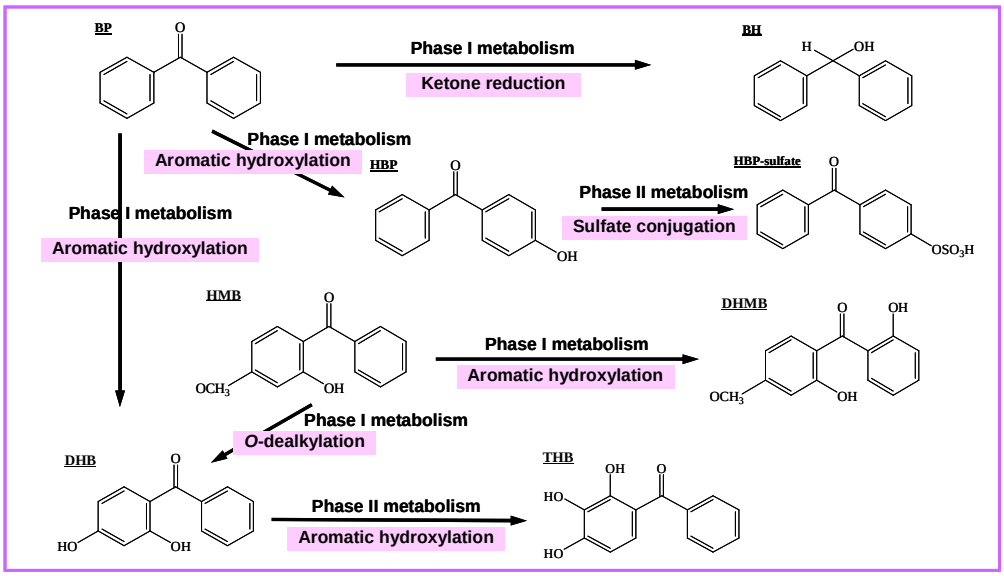


Figure 3.3. Proposed metabolism of benzophenone-type UV filters in rat hepatocytes.

Yoshio *et al*. (2002)

Chapter 4. Genotoxic Assessment of Benzophenone-Type UV Filters

4.1 Backgrounds

The alkaline version of single cell gel electrophoresis assay (comet assay) is considered to be a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells (Singh *et al*., 1988). It combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali labile sites, and cross-links, with the single cell approach typical of cytogenetic assays. In addition, the assay also provides the opportunity to estimate DNA repair kinetics in cells following exposure to genotoxic agents (Fairbairn *et al*., 1995).

The comet assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells. In this assay, a suspension of cells is mixed with lowmelting agarose and spread on a microscope glass slide. Following lysis of the cells with a detergent at high salt concentration, DNA unwinding and electrophoresis are

carried out at a specific pH. The size and shape of the comet and the distribution of DNA within the comet have been correlated with the extent of DNA damage (Fairbairn *et al*., 1995). Currently, the assay is perceived as a potentially emerging tool for genotoxicity testing and regulatory submissions (Tice *et al.*, 2000; Ryu *et al.*, 2001; Hartmann *et al*., 2003,).

Moreover, BP-type UV filters have been reported to have potential genotoxic effects (Table 4.1). The parent compound, BP, was negative in *Salmonella* mutagenesis assay (Mortelmans *et al*., 1986) and in the *Escherichia coli* pol A assay (Fluck *et al*., 1976), but HMB was mutagenic in *Salmonella* (Zeiger *et al*., 1987) and induced sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells (French, 1992). DHMB also was positive in *Salmonella* mutagenesis assay (French, 1992). Therefore, the aim of this work was to detect the different reponse of BP type UV filters in terms of their genetic damage.

4.2 Materials and Methods

4.2.1 Reagents

Methyl methanesulfonate (MMS) and benzo[a]pyrene (B[a]P) were obtained from Sigma -Aldrich Co. (St. Louis, USA). MMS was dissolved and further diluted in distilled water, and B[a]P was dissolved and further diluted in dimethyl sulfoxide (DMSO). BP was obtained from Sigma (St. Louis, MO, USA). BH, HBP, HMB, DHB, DHMB and THB were purchased from Aldrich (Milwaukee, WI, USA or St. Louis, MO, USA). The seven BP-type UV filters were dissolved in DMSO immediately before use. The final concentration of DMSO used in the medium was below 1%. RPMI-1640 medium, trypan blue and horse serum were the products of GIBCO® (California, USA). Low melting point agarose (LMPA) was a product of Amresco (Solon, OH, USA). The preparation of rat liver S-9 fraction for metabolic activation system was previously reported (Ames *et al*., 1973; Ames *et al*., 1975; Maron *et al*., 1983). The S-9 fraction prepared was stored immediately at -80℃ before use.

4.2.2. Cell Culture

The mouse lymphoma L5178Y cell line was employed for comet assay. Cells were cultivated in 90% RPMI-1640 (Life Technologies, MD, USA) with 1 mM sodium pyruvate and 0.1% pluronic, supplemented with 10% heat-inactivated horse serum and antibiotics in a humidified incubator at 37℃ with 5% CO_2.

4.2.3 Cytotoxicity Test

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, about 10^6 cells were treated for 2 hours with the chemicals. After the staining of 0.4% trypan blue (Life Technologies, MD, USA), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability of treated chemicals was related to controls that were treated with the solvent. All experiments were duplicated in an independent test.

4.2.4 Single Cell Gel Electrophoresis (Comet) Assay

4.2.4.1 Preparation of L5178Y Cells for Comet Assay

For the comet assay, 8×10^5 of cells were seeded into 12 wells plate and then treated as described in the toxicity tests. After 2 hours, cells were centrifuged for 5 min at 100 x g, and gently resuspended with PBS. 100 μl of the cell suspension was immediately used for the test. Cells were mixed with 100 μl of LMPA (1%) and added to fully frosted slide which had been covered with a bottom layer of 100 μl of 1% normal melting agarose. The cell suspension was immediately covered with coverglass and the slides were then kept at 4℃ for 5 min to allow solidification of

the agarose. After gently removing the coverglass, the slides were covered with a third layer of 100 μl of 0.5% LMPA by using a coverglass and then the slide were again kept cold at 4℃ for 5 min.

4.2.4.2 Alkaline Unwinding/Alkaline Electrophoresis

The procedure used follows the method described by Singh *et al.*(1988) with minor modification. The cells embedded in the agarose on slides were lysed for 1.5 hr in reaction mixture of 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl (pH 10), and 1% Triton X-100 at 4℃. Slides were then placed in 0.3 M NaOH and 1 mM Na_2EDTA (pH approximately 13) for 20 min to unwinding of DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4℃. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

4.2.4.3 Evaluation of DNA Damage

After the electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 5

min. The slides were stained by 50 μl of 5 μg/ml ethidium bromide solution on each slide, and then covering the slide with a coverglass. Image of 200 randomly selected cells (50 cells from each of four replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis Komet 5.5 (Kinetic Imaging Limited, Liverpool, UK), determining the mean tail moment (percentage of DNA in the tail times tail length) (Fig. 4.2) of the 200 cells. The comparison of each test group with negative and positive control was analyzed with one way of analysis of variance (ANOVA) followed by Dunn's test. $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Cytotoxicity of Benzophenone-Type UV Filters

To determine the optimal concentration, cytotoxicity of each compound was assessed by exposure to 0-5,000 μg/ml with 2-fold serial dilution for 2 hours using trypan blue exclusion assay. All measurements of cytotoxicity were performed in the presence and absence of S-9 metabolic activation systems (Fig. 4.3). Based on results of cytotoxicity assay, 20% inhibitory concentration (IC_{20}) of each compound was calculated and used as maximum concentration (Table. 4.2). HMB was shown

the highest cytotoxicity, and THB was shown the lowest cytotoxicity among all compounds tested in this study.

4.3.2 DNA Damage of Benzophenone-Type UV Filters

To investigate whether seven BP-type UV filters induce DNA strand breaks, the comet assay was performed with parent compounds (BP and HMB) and its metaoliites (BH, HBP, DHB, DHMB and THB) in L5178Y mouse lymphoma cells. Two hundred randomly selected cells per slide were analyzed, and the genotoxic effect of seven BP-type UV filters on the L5178Y cell nuclei DNA damage was measured using Komet 5 imaging software.

The L5178Y cells were treated with seven BP-type UV filters for 2 hr and at least four slide (800 cells) were analyzed for each sample. A clear induction of DNA strand breakage was observed after 2 hr positive control treatment. The response of the positive control (150 μM MMS and 50 μm B[a]P) was significantly greater ($p < 0.05$) than negative control in condition without or with S9 metabolic activation system, respectively.

Fig. 4.4 (a-c) shows the tail moment of BP and its metabolites, HBP and BH in mouse lymphoma L5178Y cells. From the results, BP and its metabolite, HBP

were observed statistically significant differences of tail moment values compared with negative control ($P < 0.05$), except for BH. In detail, the DNA damaging effects of BP were assessed at concentration from 12.5 to 50.0 μg/ml in the presence of S9 metabolic activation systems (+ S9) and from 18.0 to 72.1 μg/ml in the absence of S9 metabolic activation systems (- S9). BP at tested all concentrations was revealed significant difference of tail moment compared to negative control in -S9. In +S9, BP at 25.0 and 50.0 μg/ml was induced a significant DNA damage ($P < 0.05$) (Fig. 4.4, a). HBP was assessed at concentration from 20.7 to 83.0 μg/ml in + S9 and from 24.3 to 97.0 μg/ml in -S9. HBP was induced DNA damage at 41.5-83.0 and 48.5-97.0 μg/ml in the +S9 and -S9, respectively ($P < 0.05$) (Fig. 4.4, b). And also, BH was performed at concentration from 16.6 to 66.3 μg/ml in +S9 and from 13.8 to 55.1 μg/ml in -S9. BH at tested all concentrations was not observed significant difference of tail moment compared to negative control in -S9 and +S9 (Fig. 4.4, c)

The tail moment of HMB and its metabolites, DHB, DHMB and THB was shown in Fig. 4.5 (a-d). From the results, HMB and its metabolite, DHB and THB were revealed statistically significant differences of tail moment values compared with negative control ($P < 0.05$), except for DHMB. In detail, the DNA damaging effects of HMB were assessed at concentration from 11.0 to 44.3 μg/ml in +S9 and

from 11.4 to 45.7 μg/ml in -S9. HMB at tested all concentrations was revealed significant difference of tail moment compared to negative control in +S9. In -S9, HMB at 45.7 μg/ml was induced a significant DNA damage ($P < 0.05$) (Fig. 4.5, a). DHB was assessed at concentration from 17.5 to 69.9 μg/ml in +S9 and from 18.8 to 75.1 μg/ml in -S9. DHB was induced significant DNA strand breaks at 34.9-69.9 and 75.1 μg/ml in the +S9 and -S9, respectively ($P < 0.05$) (Fig. 4.5, b). And also, DHMB was performed at concentration from 13.1 to 52.4 μg/ml in +S9 and from 12.9 to 51.5 μg/ml in -S9. DHMB at tested all concentrations was not obserbed significant difference of tail moment compared to negative control in -S9 and +S9 (Fig. 4.5, c). The DNA-damaging effect of THB was assassed at 31.0 to 123.9 μg/ml in +S9 and at 29.3 to 117.3 μg/ml in -S9. THB at tested all concentrations was shown significant DNA damage in -S9. In +S9, THB was induced significant DNA strand breaks at 61.9-123.9 μg/ml ($P < 0.05$) (Fig. 4.5, d).

4.4 Discussion

It is well known that carcinogenicity is the most serious effect of toxic chemicals in human health. One of the mechanisms of carcinogenicity, induction of DNA damage can be determined by comet assay, which is widely used for the detection and measurement of DNA strand breaks (Singh *et al*., 1988; Tice *et al*.,

1991; Fairbairn *et al*., 1996; Anderson *et al*., 1998; Speit *et al*., 1999). The main advantages of the comet assay include: (a) the collection of data at the level of the individual cell, allowing more robust statistical analyses; (b) the need for only a small number of cells per sample (<10,000); (c) sensitivity for detecting DNA damage; and (d) possible use of any eukaryote single cell population, including cells obtained from natural biota or aquatic organisms for eco-genotoxicological studies and environmental monitoring (Dixon *et al*., 2002; Lee *et al*., 2003).

In this respect, to investigate whether seven BP-type UV filters induce DNA strand breaks, the comet assay was performed with parent compounds (BP and HMB) and its metaoliites (BH, HBP, DHB, DHMB and THB) in L5178Y mouse lymphoma cells following guideline recommended by International Workshop on Genotoxicity Test Procedures (IWGTP) (Tice *et al*., 2000).

In the case of BP and its metabolites, BP and HBP were observed statistically significant DNA damage ($P < 0.05$), except for BH. BH was not observed significant difference of tail moment compared to negative control. In the case of HMB and its metabolites, HMB and DHB and THB were revealed statistically significant DNA damage, except for DHMB. DHMB was not induced significant DNA strand breaks at tested all concentrations.

Therefore, we suggest that BP derivatives with hydroxyl group at the *para*-position such as HBP, DHB and THB possibly possess the single stranded DNA breakage in mouse lymphoma cell line.

Table 4.1. Toxicity data of benzophenone-type UV filters

Name	Ames test	MOLY	CA	Micronuclei
BP	negative	negative	-	not induce
BH	-	-	-	-
HBP	-	-	-	-
HMB	positive (+S9)	positive (+S9/ -S9)	weakly positive (S9)	not induce
DHB	negative	-	-	-
DHMB	TA1537 positive (+S9)	-	-	-
THB	-	-	-	-

National Toxicology program, 2000

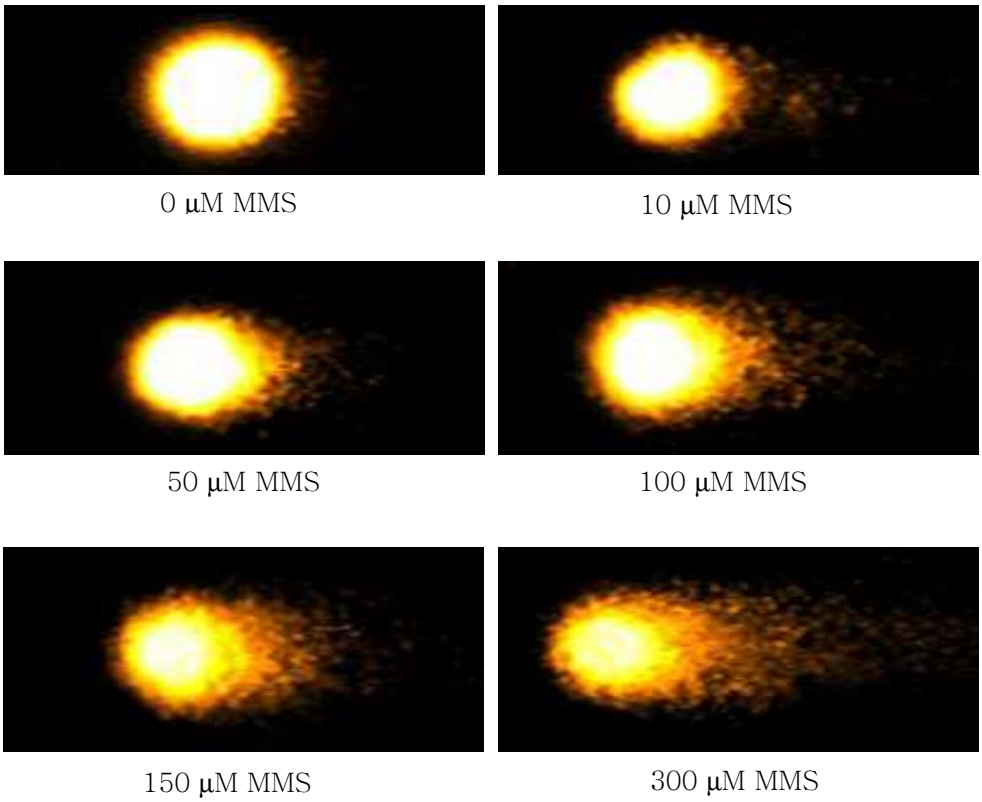


Figure 4.1. Typical DNA migration patterns in mouse lymphoma L5178Y cells by comet assay.

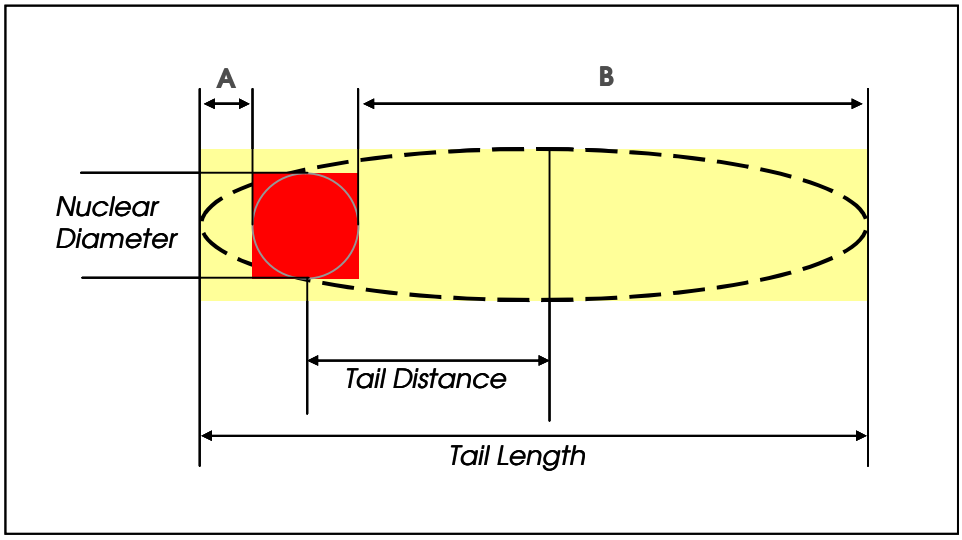
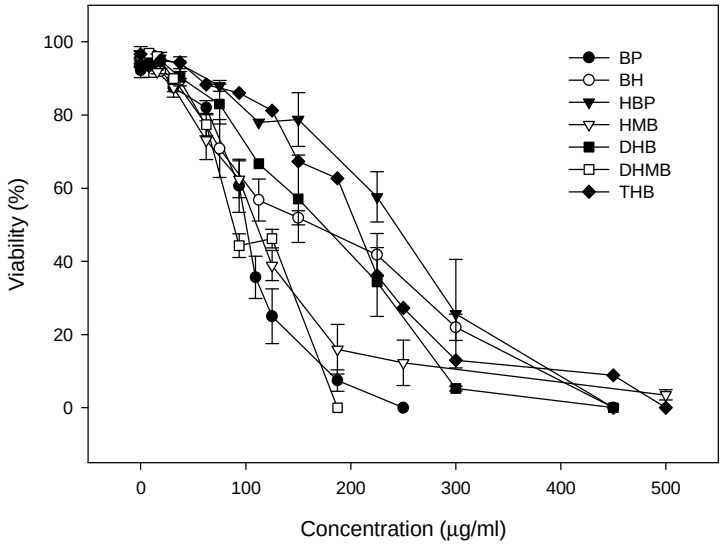


Figure 4.2. A definition of parameter of comet assay.

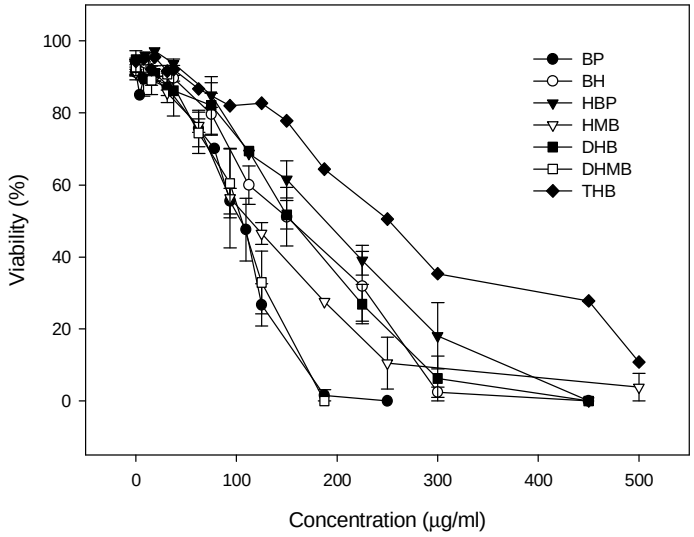
A + B = DNA migration

Tail distance = center position of tail − center position of head

Tail moment = Tail distance × % DNA in Tail



(a) The absence of S9



(b) The presence of S9

Figure 4.3. Cytotoxicity tests of benzophenone-type UV filters by trypan blue dye exclusion assay in L5178Y cells.

The trypan blue dye exclusion assay was carried out at least in triplicate.

Table 4.2. 20 % Inhibitory concentration values of benzophenone-type UV filters in L5178Y cells

Chemicals	IC_{20} (μg/ml)	
	-S9	+S9
BP	72.10	50.04
BH	55.14	66.32
HBP	97.04	82.96
HMB	45.72	44.29
DHB	75.14	69.87
DHMB	51.50	52.40
THB	117.33	123.88

The experimental values are means of three independent experiments.

IC_{20} = 20 % Inhibitory concentration

-S9 = The absence of S9; +S9 = The presence of S9

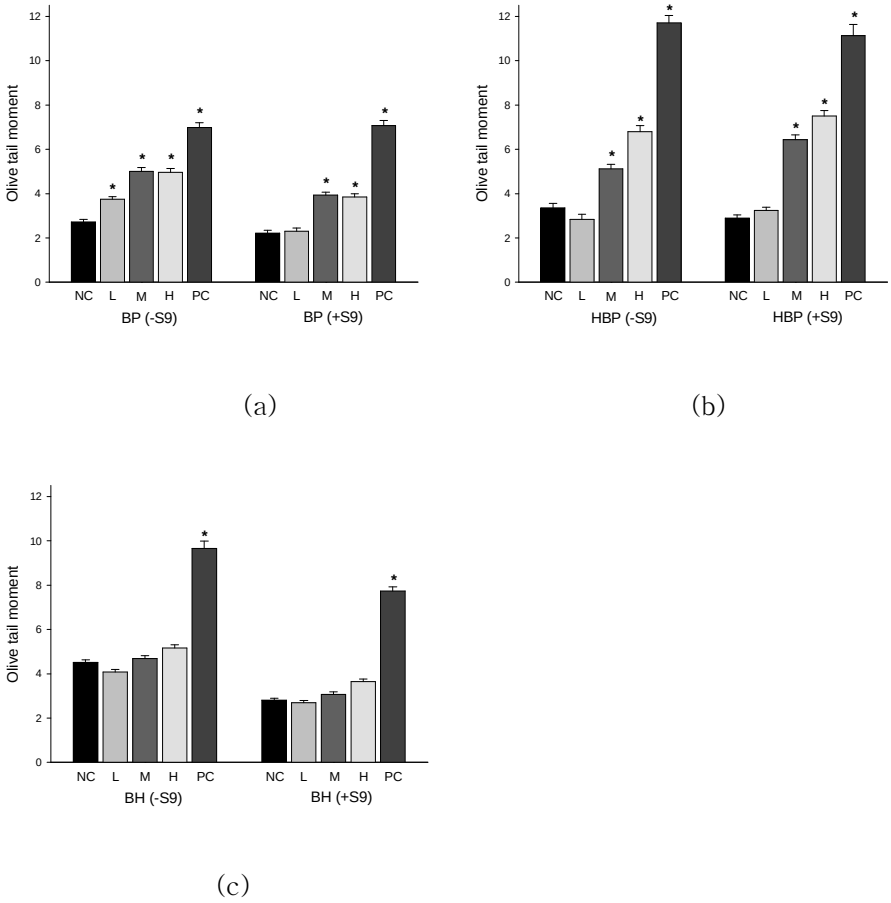


Figure 4.4. DNA damages by benzophenone (a), 4-hydroxybenzophenone (b) and benzhydrol (c) in L5178Y cells.

Values are means ± SE from four experiments. In each experiement tail moment index had been assessed from 200 separately calculated cells.

-S9 = The absence of S9; +S9 = The presence of S9; NC =Negative control (DMSO); PC = Positive control (-S9, MMS 150μM, +S9, B[a]P 50 μM); Significance (*) = $P < 0.05$

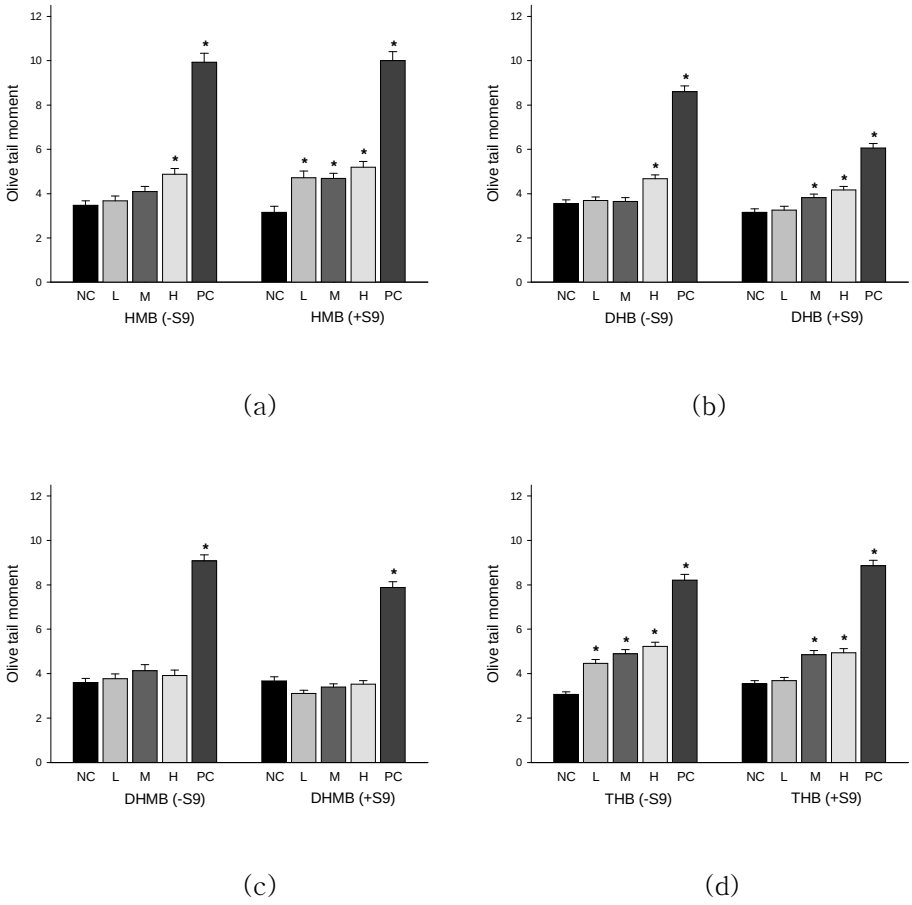


Figure 4.5. DNA damages by 2-hydroxy-4-methoxybenzophenone (a), 2,2'-dihydroxybenzophenone (b), 2,2'-dihydroxy-4-methoxy benzophenone (c) and 2,3,4-trihydroxybenzophenone (d) in L5178Y cells.

Values are means ± SE from four experiments. In each experiement tail moment index had been assessed from 200 separately calculated cells.

-S9 = The absence of S9; +S9 = The presence of S9; NC =Negative control (DMSO); PC = Positive control (-S9 = MMS 150μM, +S9 = B[a]P 50 μM); Significance (*) = $P < 0.05$

Chapter 5. Toxicogenomic Assessment of Benzophenone-Type UV Filters

5.1 Backgrounds

Toxicogenomics, in a broader sense, is defined as a study of the response of a genome to hazardous substances, using: i) genomic-scale mRNA expression (transcriptomics) ii) cell and tissue wide protein expression (proteomics), and iii) metabolite profiling (metabon/lomics) in combination with bioinformatics methods and conventional toxicology (In a narrow sense, it refers to the use of transcriptomics) (Ryu *et al.*, 2005). In relation to chemical hazard/risk assessment, this emerging science could provide tools for improving the understanding of mechanism of toxicity, identification of biomarkers for prediction of toxicity and exposure, and possibly alternative methods for chemical screening, hazard and toxicity identification, characterization, classification. The impact of human genome projects on toxicological research is high, heralding the emerging technologies of toxicogenomics, proteomics, and bioinformatics (Lovett, 2000; Pollack, 2000) for the future use of these technologies and their impact on drug discovery, safety evaluation, elucidation of pathways of toxicity, and risk assessment. The National Institute of Environmental Health Sciences (NIEHS)

established the National Center for Toxicogenomics (NCT) in September 2000. According to the center's mission statement, its goal is "to use the methodologies and information of genomics science to significantly improve our understanding of basic biological responses to environmental stressors/toxicants."

BP has been listed among "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund and the Japanese Environment Agency (NIES, 1998). In the previous studies, a possible estrogenic activity of BP-type UV fitlers was suggested based on the observation that BP-type UV fitlers induced cell proliferation in MCF-7 breast cancer cells transfected with the estrogen receptor (Nakagawa *et al*., 2000, 2002) and increased uterine weight in juvenile female rats and in ovariectomized rats exposed to doses of 400 mg/kg (Nakagawa *et al*., 2001, 2002).

Currently, a wide variety of testing methods have been developed to investigate endocrine disrupting chemicals (EDCs) (Jobling et al., 1998). These include physical and chemical fractionation methods (Huang and Sedlak, 2001; Smeds and Saukko, 2001; Sole et al., 2000), the study of biomarkers in sentinel species, and single mode of action (MOA) oriented *in vivo* and *in vitro* assays, and life cycle or multigenerational *in vivo* tests. Development of novel approaches for high-throughput screening for potential EDCs is a major goal in the environmental

health research. The need for advancing prediction the adverse biological effects of EDCs in mammalian has made technologies exploiting advances in molecular techniques. Current molecular-level techniques rely on ligand-binding assays (Takeyoshi et al., 2002), enzyme-linked immunosorbent assay (ELISA) (Nilsen et al., 2004), and more recently, gene expression profiling (Imanishi et al., 2003; Nielsen et al., 2000). In the near future, more reliance will be placed on the development of gene expression assays to determine the intricate interactions between genes that are affected by the exposures. It has the potential to implicate previously unsuspected estrogen, androgen and thyroid hormone-sensitive genes that may later become molecular markers of endocrine disruption (Inoue, 2003; Terasaka et al., 2004).

In this respect, we designed an in-house cDNA microarray system, namely KISTCHIP-400, which contains 416 human genes known or proposed to be involved in endocrine system, containing estrogen, androgen, thyroid hormone, endocrine regulation and homeostasis (Ryu and Kim, 2005). It may be assisted to identify the potential EDCs and to understand molecular toxicological mechanisms of EDCs. Our laboratory had involved in the safety assessment of environmental hazardous chemicals. We outline an experimental system with higher sensitivity for

toxicogenomic evaluation of estrogen activity in natural and industrial chemicals on the basis of statistical analysis of gene response.

Therefore, in the present study, to identify genes elicited by three UV filters of BP-type, we carried out a human cDNA microarray analysis of MCF-7 breast cancer cell line, treated with BP, HBP and HMB using KISTCHIP-400 including 401 endocrine system related genes.

5.2 Materials and Methods

5.2.1 Cell Cultures

MCF-7 cell line originated from human breast cancer cell was a gift from the Prof. Soto (Tufts University School of Medicine, Boston, MA, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen Life Technologies) supplemented with 5% fetal bovine serum (Invitrogen Life Technologies), penicillin 100 IU/ml and streptomycin 100 ㎍/ml. Subculturing was conducted every 3 day so as not to exceed 1×10^6 cells/ml.

5.2.2. Cell Proliferation Assay (E-Screen Assay)

MCF-7 cell line was tested with slight modification described by Soto et al., (1995). Cells were trypsinized and plated in 12-well plates at an initial concentration of 3×10^4 cells per well in 5% FBS in DMEM. The cells were allowed to attach for 24 h, then 5% charcoal dextran-treated fetal bovine serum (CDFBS) supplemented phenol red-free DMEM was substituted for the seeding medium. Appropriated concentrations of the test compounds were added. The assay was stopped after 6 days by mixing with 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis MO) and the plate was further incubated for 3 h at 37℃. The medium was removed and the formed formazan crystals were dissolved with 1 ml of DMSO. Finally, the aliquots were transferred to a 98-well plate to read optical density (OD) in a microtiter plate reader at 540 nm. Linearity of optical density was evaluated in the MTT assay (Soto et al., 1995; Mosmann, 1983).

5.2.3 RNA Extraction

At 80% confluence, cells were treated with 10^{-8} M E2 (Sigma, St. Louis MO), 10^{-4} M BP, 10^{-5} M HBP and 10^{-5} M HMB in phenol-red-free DMEM/F12 supplemented with 5% CDFBS culture media for 48 h and these chemicals were dissolved in DMSO. Vehicle concentrations were less than 0.1% in all experiments. Total RNA was isolated from MCF-7 cells with E2 and three BP-type UV filters

using Trizol reagent (Invitrogen Life Technologies) and purified using RNeasy mini kit (Qiagen, CA) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen, CA) during RNA purification. The amount of each total RNA sample was measured by a spectrophotometer, and its quality was checked by agarose-gel electrophoresis.

5.2.4 cDNA Microarray

We examined transcriptional profiling of E2, BP, HBP and HMB. For cDNA microarray analysis, total RNA was isolated from MCF-7 cells with each four chemical treatment. Labeling and hybridization were performed by instruction of MICROMAX direct cDNA microarray system (Perkin Elmer Life Sciences, MA) with minor modification. Briefly, the RNA samples from MCF-7 cells of treated chemicals were labeled with Cy3-dUTP (NEN, MA), and those of non-treated chemicals were labeled with Cy5-dUTP (NEN, MA). The two colour probes were then mixed, purified using Microcon YM-100 column (Millipore, MA). Hybridization and washes were performed according to the Digital Genomics Inc.'s (Seoul, Korea) instruction. Hybridization (hybridization buffer; 25% formamide, 5× SSC, 0.1% SDS, 0.5 mg/ml polyA, 0.5 mg/ml Cot-1 DNA) was performed in a hybridization oven at 58℃ for 16 h. After washing (2× SSC/0.1% SDS for 5 min at 58℃, 0.1× SSC/0.1% SDS for 10 min at RT, 0.1× SSC for 1 min at RT), the

slide was dried by centrifugation at 650 rpm for 5 min. Hybridization images on the slides were scanned by ScanArray Lite (PerkinElmer Life Sciences, MA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, CA) to obtain gene expression ratios. The overall intensities were normalized using a correction coefficient obtained from the ratios of housekeeping genes.

5.2.5 Quantitative Real-Time RT-PCR

We used the same mRNA pools for both microarray and real-time RT-PCR. mRNA quantification was performed using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). The mRNAs were reverse-transcribed into cDNAs by using an Omniscript RT kit (Qiagen, CA). The Primer specificity was tested by running a regular PCR for 40 cycles at 95°C for 20 s and 60°C for 1 min, and followed by an agarose gel electrophoresis. The real-time PCR was performed by using a SYBR supermix kit (Bio-Rad, Hercules, CA), and running for 40 cycles at 95°C for 20 s and 60°C for 1 min. The PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. Each cDNA sample was triplicated and the corresponding no-RT mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The mRNA level of each sample for each gene was normalized to that of the β-actin mRNA. Relative mRNA level

was presented as $2^{[(Ct/\ \beta\text{-actin} - Ct/\text{gene of interest})]}$. All data shown were the mean ± SD of three separate experiments. For quantitative PCR, primers used were as Table 5.2.

5.2.6 Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, WA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes (Tusher et al., 2001). The statistical significance of the differential expression of genes was assessed by computing a *q*-value for each gene. To determine the *q*-value we used a permutation procedure, and for each permutation, two-sample t statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in four independent hybridizations were more than 0.65 or less than -0.65, i.e., 1.5-fold difference in expression level, and when the *q*-values were <5.

5.3 Results

5.3.1 Estrogenic Activity and Dose Selection of Benzophenone-Type UV Filters

E2 at concentrations ranging from 10^{-11} to 10^{-5} M was used as a reference chemical, with known estrogenic effects. The relative cell numbers were measured at 6 day of treatment with BP, HBP and HMB at concentrations from 10^{-9} to 10^{-3} M. The effects of the test chemicals on the proliferation of MCF-7 cells are shown in Fig. 5.1. A 1.5-fold increase in cell growth over the control was adopted as a criterion of estrogenic. All chemicals tested significantly increased MCF-7 cell growth in the E-screen. Significant proliferation of MCF-7 cells was induced by E2 at concentration of 10^{-8} M ($p < 0.05$). In order to examine microarray for BP, HBP and THB, we were chosen the 10^{-4}, 10^{-5} and 10^{-5} M concentration with the highest estrogenic potency dose, respectively.

5.3.2 Analysis of the Gene Expression Patterns Induced by Benzophenone-Type UV Filters

To evaluate whether genes spotted upon constructed cDNA microarray responds to EDCs as three BP-type UV filters with estrogenic activity, 10^{-4} M BP, 10^{-5} M HBP and 10^{-5} M HMB was treated to observe genes and its expression profile. Five independent experimental samples for each treatment group were analyzed to determine RNA transcript levels. Based on the number of genes expressed in control versus treated samples, as well as on the level of expression of individual genes, the overall gene expression pattern was similar between control

(vehicle-treated) and estrogenic compound-treated (E2, BP, HBP and HMB) (Fig. 5.2). Although the number of genes whose expression is altered by BP, HBP and HMB is not very big, there are many genes whose expression is modified by exposure to each compound. Of the 416 genes, there were 38, 38 and 30 genes whose expression level showed some evidence of treatment effect (up- or down-regulated) by BP, HBP and HMB, respectively, relative to their respective vehicle control and judged by fold change (at least ± 1.5 fold, up or down)(Fig. 5.2). In the conmmon genes of BP, HBP and THB, 4 genes were activated and 7 genes were repressed. The common genes for E2 were higher HBP than BP and HMB (HBP, 25 > BP, 18 > HMB, 8). These results suggest that estrogenicity of HBP was the most similar with E2. Also, analysis of the data derived from E2, BP, HBP and HMB, indicated that the expression of 7 genes was consistently and significantly regulated in the same direction, although at a different magnitude (Fig. 5.3). These include genes known to be directly regulated by estrogens but also other annotated genes and ESTs that have not been previously identified as estrogen-responsive one. Table 5.3 shows the complete list of the 7 genes from our studies that showed a statistically significant change in their expression by estrogenic exposure, along with their accession number. Among the groups of genes, there was significant up-regulation of the genes related to cell proliferation, regulation of tyroid hormone, metabolism, transcription and transport.

4.4 Discussion

Toxicogenomic approach will be powerful tools (Ryu et al., 2005) and may solve the biological relevance of very low quantity with long term exposure of environmental hazardous chemicals at present.

A significant concern has recently been raised about the potential of environmental chemicals that might disrupt endocrine function. Particular attention has been given to chemicals that are able to alter estrogen functions. For this reason, we evaluated whether exposure to estrogenic compounds could be identified by transcripts profiling. In this study we have used this approach to identify the gene expression profile induced by 4 chemicals with estrogenic activity, E2, BP, HBP and HMB, in MCF-7 cells.

An increased awareness of EDCs and their potential to affect wildlife and humans has produced a demand for practical screening methods to identify endocrine activity (Gaido et al., 1997; Skakkebaek, 2002). Despite the exciting prospects of this methodology, a scan of the literature reveals very few toxicogenomic studies. In our previous report, we have constructed a human endocrine related cDNA microarray, called KISTCHIP-400, which contains many of the human genes known or proposed to be involved in endocrine system (Ryu

and Kim, 2005). We believe will serve as a template for future studies in toxicogenomics for performing EDCs monitoring. Through this kind of studies, it is possible to identify the patterns of alteration of gene expression characteristic of exposure to estrogen like EDCs in cultured human cells. And once signatures are identified, the patterns of altered gene expression induced by unknown agents might identify their mechanism of action. This approach could also be applied to identify signatures for various types of tissue-specific EDCs, providing a more rapid test for the possible toxicological effect of drugs or unknown agents and less need to use animals.

Table 5.1. Estrogenic activity of benzophenone-type UV filters

Chemiclas	MCF-7 cell Assay (M)	Competitive binding assay (IC_{50}, M)	Uterotropic assay (mg/kg/day)	Yeast two hybrid Assay REC_{10} (M)
BP	$> 10^{-4}$	$> 5 \times 10^{-4}$	> 400	$> 1.0 \times 10^{-3}$
BH	$> 10^{-4}$	$> 5 \times 10^{-4}$		
HBP	10^{-5} -10^{-4}	5×10^{-5}	100-400	4.5×10^{-6}
HMB	> 10-^{5}	$> 1 \times 10^{-4}$	ED_{50} 1,000-1,500	6.6×10^{-4}
DHB	10^{-8} -10^{-6}	5×10^{-5}	positive	1.8×10^{-6}
DHMB	10^{-7} -10^{-6}	$> 1 \times 10^{-4}$		1.0×10^{-3}
THB	10^{-7} -10^{-6}	5×10^{-4}		9.0×10^{-6}

Table 5.2. Primer sequences used for quantitative RT-PCR of response genes by 17β-estradiol and benzophenone-type UV filters

Genes	Accession No.	Primer Sequences	Product (bp)
SERPINA7	T64901	F: 5'- TTGTGAACCCAACGGAAGCGTAGT-3' R: 5'- TTCCACAGCCAACAAAGTTCAGCC-3'	175
MGP	AA155913	F: 5'-AGAGCTAAAGTCCAAGAGAGGATCCGAG-3' R: 5'-CGTAGCGTTCGCAAAGTCTGTAGTCA-3'	97
TPD52L1	NM_003287	F: 5'-CAACTGTCACAAGCCTCAAGA-3' R: 5'-AGCCTCCTGCCAAGCTCT-3'	97
CYP27B1	AI222585	F: 5'-AGGGACAGGCCCAAAGAT-3' R: 5'-CTGGCCACTATTAAGTGGGTTT-3'	48
CYP2E1	J02625	F: 5'-ACTCCCTGGCTCCAGCTTTACAAT-3' R: 5'-TGCTCCTTCACCCTTTCAGACACA-3'	125
EGR1	AA486628	F: 5'-GCCTGCGACATCTGTGGAA-3' R: 5'-CGCAAGTGGATCTTGGTATGC-3'	71
DIO3	N67048	F: 5'-AAGCGCCTCAAACCAAGTC-3' R: 5'-GGTGGCTCAGCACATCAGT-3'	43
MYCN	R66447	F: 5'-CGCAAAAGCCACCTCTCATTA-3' R: 5'-TCCAGCAGATGCCACATAAGG-3'	96
PGAM1	J04173	F: 5'-TCAAGGAGGGGAAACGTGTA-3' R: 5'-GGTTCAGCTCCATGATAGCC-3'	105
MDH1	D55654	F: 5'-CTGCTCTACTCATTCCCTGTTGT-3' R: 5'-CATTAATAGGGAGACCTTCAACAAA-3'	46

SERPINA7; Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7, MGP; Matrix Gla protein, TPD52L1; Tumor protein D52-like 1, CYP27B1; Cytochrome P450, family 27, subfamily B, polypeptide 1, CYP2E1; Cytochrome P450, family 2, subfamily E, polypeptide 1, EGR1; Early growth response 1, DIO3: Deiodinase, iodothyronine, type III, MYCN; V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian), PGAM1; Phosphoglycerate mutase 1 (brain), MDH1; Malate dehydrogenase 1, NAD (soluble).

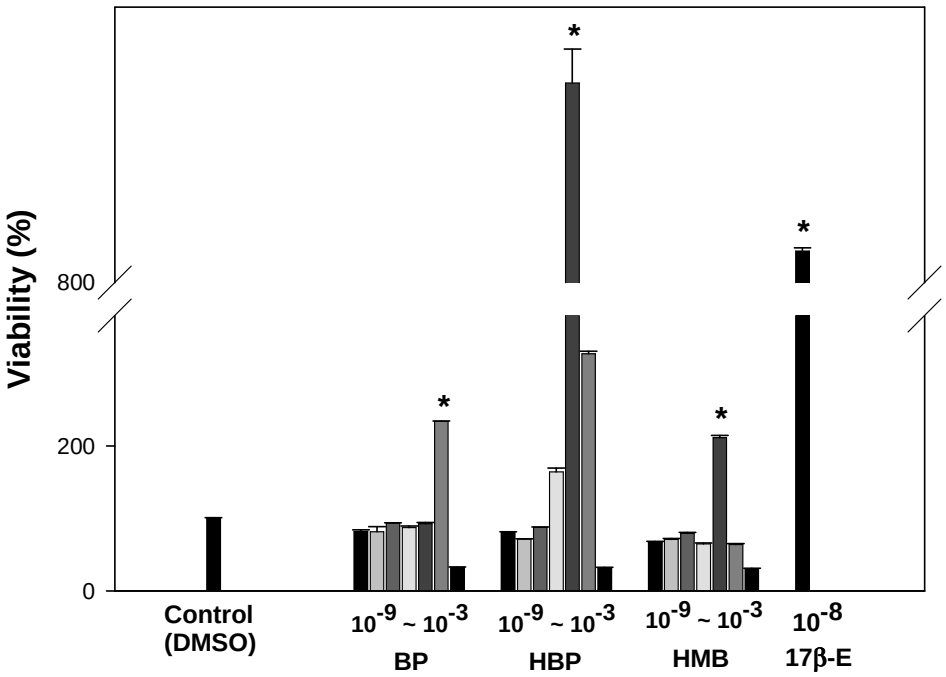


Figure 5.1. Cell proliferation of 17β-estradiol, benzophenone, 4-hydroxy benzophenone and 2-hydroxy-4-methoxybenzophenone by E-screen assay.

Cells were exposed to test compounds for 6 days with 5% charchol/dextran-treated serum in the cell culture medium. Asterisks indicate group means that were significantly different from the control group.

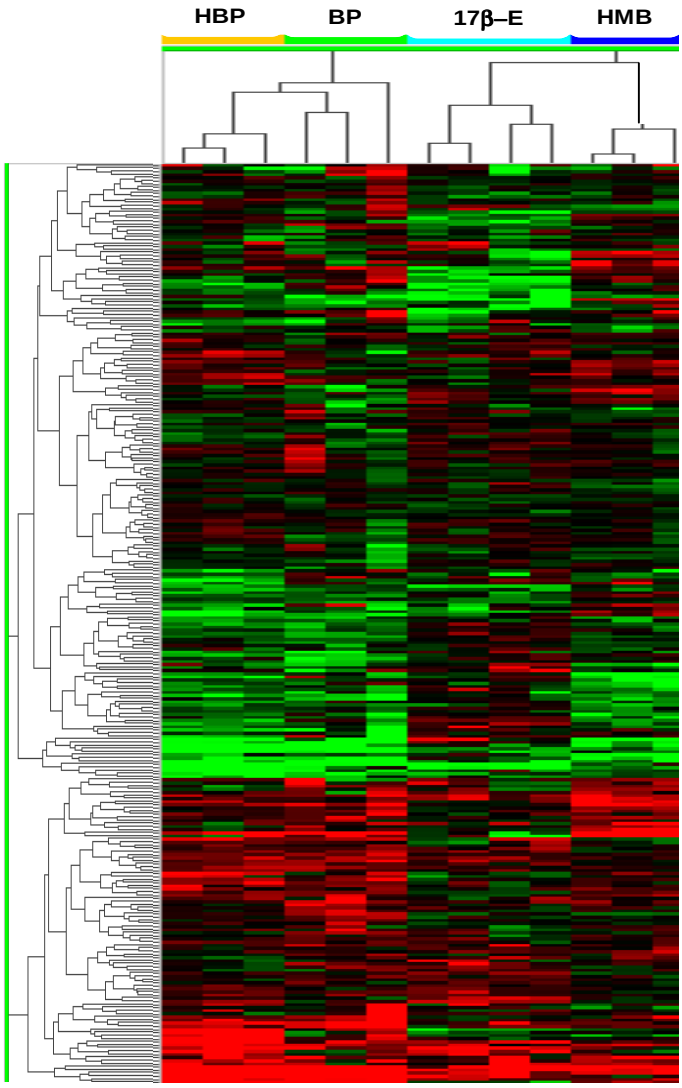
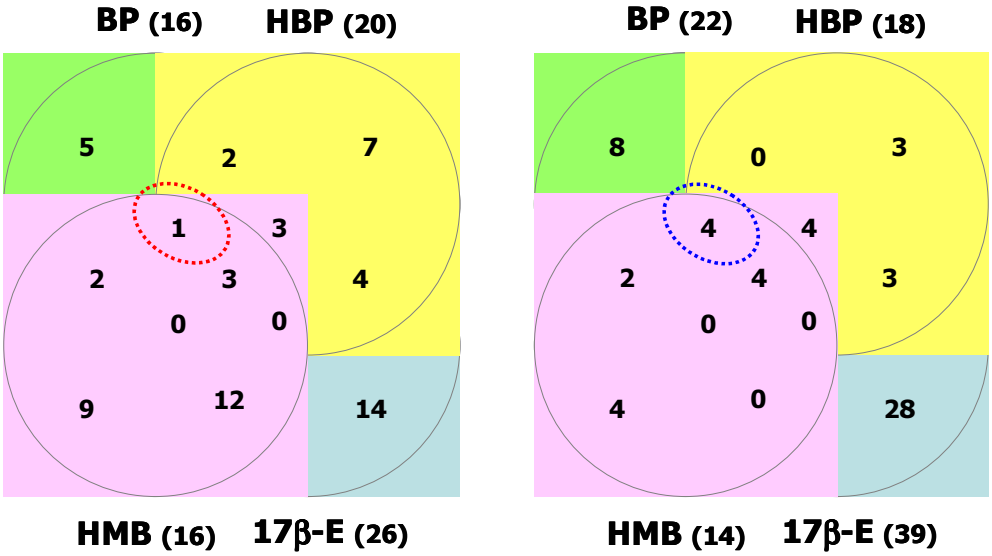


Figure 5.2. Cluster Analysis of benzophenone-type UV filters induced expression profiles in MCF-7 Cells.

Cells were treated with 10^{-4} M benzophenone (BP), 10^{-5} M 4-hydroxybenzophenone (HBP), 10^{-5} M 2-hydroxy-4-methoxybenzophenone (HMB) and 10^{-8} M 17β-estradiol. Transition of color for each gene from black to green indicates a gradual decrease in expression with time, and from black to red indicates up-regulation of gene expression.



(a) Up-regulated genes **(b) Down-regulated genes**

Figure 5.3. Venn diagrams of estrogen-responsive genes.

Venn diagrams were created using genes with a differential expression of at least ± 1.5 fold, up- or down-regulated in cells treated with 17β-estradiol (E2), benzophenone (BP), 4-hydroxybenzophenone (HBP) and 2-hydroxy-4-methoxy-benzophenone (HMB) compared with vehicle-treated control cells.

Table 5.3. List of up- and down-regulated genes by 17β-estradiol and benzophenone-type UV filters

Accession No.	Gene title	Symbol	Gene functions
Up-regulated genes			
AA155913	Matrix Gla protein	MGP	structural constituent of bone
NM_003287	Tumor protein D52-like 1	TPD52L1	breast carcinoma associated ;cell proliferation
AI222585	Cytochrome P450, family 27, subfamily B, polypeptide 1	CYP27B1	Metabolism ;calcium metabolism ;tissue differentiation
T64901	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	SERPINA7	thyroid hormone transport protein
Down-regulated genes			
N67048	Deiodinase, iodothyronine, type III	DIO3	selenoprotein, regulation of thyroid hormone inactivation
AI021885	Protein C (inactivator of coagulation factors Va and VIIIa)	PROC	Immune System, ;blood coagulation
AA323489	Aminolevulinate, delta-, synthase 1	ALAS1	Metabolism ;amino acid metabolism
J04173	Phosphoglycerate mutase 1 (brain)	PGAM1	Metabolism ;carbohydrate metabolism
AA486628	Early growth response 1	EGR1	transcriptional regulator
AA670429			unknown
R66447	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	MYCN	transcription factor
J02625	Cytochrome P450, family 2, subfamily E, polypeptide 1	CYP2E1	Metabolism ;lipid metabolism

Through the clustering analysis of gene expression profiles, we identified 4 up-regulated and 8 down-regulated common genes changed by three UV filters. Among the genes, especially, 3 genes were induced and 3 genes were repressed by three UV filters of BP-type as 17β-estradiol. The shadow lines were only presented common regulated gene by three BP-type UV filters

Chapter 6. Overall Conclusions

Although the amounts of BP-type UV filters used are small compared to those of many other chemicals, the environmental consequences may still be significant, due to the possibie everyday direct input of human. Especially, exposure of BP-type UV filters to susceptible individuals, such as developing embryo and fetus, may be happened significant toxicological problem. Therefore, in this thesis, we focused on the potential risk and overall toxicity of the BP-type UV filters in environment, animal, cell and gene levels using the various toxicological tools.

First, we investigated: (i) the development of a suitable analytical method to quantify the seven BP-type UV filters in environmental samples by GC-MS and (ii) determination of the concentrations of these UV filters in real water and soil samples between April and May 2003, Korea. A novel method has been developed to simultaneously quantify seven UV filters employing liquid (solid)-liquid extraction, derivatization with MSTFA using GC-MS in environmental matrices. Under optimal conditions, good linearity for each UV filter obtained and the MDL and MQL were relatively low, below the 100 and 500 ng/L or kg, respectively. The high recovery and the low RSD values for water and soil samples indicated the high

performance of this method. From this validated method, the overall concentration of UV filters in the soil samples (500-18,380 ppt) was highly distributed in water samples (27-204 ppt). The major water contamination source of of BP-type UV filters were derived from the industrial wastewater better than municipal and recreational water. Moreover, despite the fact that the initial contamination of the UV filters happens directly through municipal, recreational water and industrial wastewater, the secondary contamination is thought to happen through the ground soil and sediment.

Secondly, it is the purpose of this study (i) to develop a suitable analytical method to quantify the seven BP-type UV filters in plasma samples by GC-MS and (ii) to determin the BP and HMB metabolism and kinetic behavior in rats after oral administration. Plasma sample was extracted with ethyl acetate (pH 9.5) and the extract was reduced to dryness, derivatized with MSTFA and determined by GC-MS. Under optimal conditions, calibration curves showed a good linearity, and the recovery, intra- and inter-day RSD, and RME were within the acceptable values, indicating good accuracy and precision. From this validated method, the major metabolites of BP were identified BH and HBP in rat. Also, HMB was enzymatically converted DHB, DHMB and THB. The toxicokinetic parameters were presented that the C_{max} value of BP was 2 μg/ml and the T_{max} was 4 hr. The $t_{1/2}$

of BP was approximately 19 hr and the $AUC_{0-\infty}$ was 47.17 ㎍/ml.hr. The C_{max} value of HMB was 21 μg/ml and the T_{max} was 3 hr. The $t_{1/2}$ of HMB was approximately 4.58 hr and the $AUC_{0-\infty}$ was 104.89 ㎍/ml.hr. The results represented clearly indicate rapid absorption of BP and HMB from the gastrointestinal tract. Structurally BP and HMB is a diphenyl ketone. The presence of two aromatic rings in its molecule confers some degree of lipophilicity to the compound, thus allowing faster absorption of the chemical from the gastrointestinal tract and faster disappearance (biphasic parrern) from plasma. The disappearance from plasma may be due to redistribution to compartments other than blood.

Thirdly, the aim of this work was to identify the different reponse of BP-type UV filters in terms of their genotoxic effects. To evaluate the magnitude of DNA damage, we used the single cell gel electrophoresis (comet) assay in the mouse lymphoma L5178Y cells. From these results, BP and its major metabolite, HBP were observed significant DNA damage compared with negative control (NC). Also, HMB and its metabolite, DHB and THB were revealed significant differences of tail moment values compared with NC. Therefore, we suggest that BP derivatives with hydroxyl group at the *para*-position such as HBP, DHB and THB possibly possess the single stranded DNA breakage in L5178Y cells.

Finally, in the present study, to identify genes elicited by three BP-type UV filters, we carried out a human cDNA microarray analysis of MCF-7 human breast cancer cells using KISTCHIP-400 including 401 endocrine system related genes. Through the clustering analysis of gene expression profiles, we identified 4 up-regulated and 6 down-regulated common genes changed by three BP-type UV filters. Among the genes, especially, 3 genes were induced and 3 genes were repressed by three BP-type UV filters as 17β-estradiol. In the further study, we will confirm the gene expression profiles identified from microarray analyses, and examined the expression patterns of the other UV filters with estrogenic activity by dose- and time- dependent manner using real time RT-PCR. Taken together, this study will be find the potential marker genes for estrogenic effect of BP-type UV filters and specific-regulated new biomarker genes by BP-type UV filters in MCF cells.

In conclusion, this thesis represents significant effects for exposure of BP-type UV filters viewed from different angles using toxicological tools. Based on the above results, this study provides basic information for risk assessment of BP-type UV filters and then it should be established as a criterion of regulation for use and addition of BP-type UV filters in environmental, food and other product.

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국 문 요 약

벤조페논계 자외선차단제는 주로 방향강화제나 살충제, 농약, 의약품 제조의 원료로 사용되어지며, 플라스틱, 잉크 등의 첨가제로서 사용되고 있다. 특히 2-하이드록시-4-메톡시벤조페논은 2,2‘-다이하이드록시-4-메톡시벤조페논과 함께 화장품, 자외선차단로션, 립스틱, 모발 염색제, 샴프 및 세제 등에서 자외선안정제의 기능성 원료로서 많이 사용되고 있다. 그러므로, 본 연구에서는 벤조페논계 자외선차단제의 환경 중 노출 수준과 대사작용, 동력학적 동태, 유전독성, 특이적으로 조절되는 지표유전자 도출에 이르기까지 전반적인 독성학적 접근법을 이용하여 연구를 수행하였고, 그 결과는 다음과 같다.

첫째로, 환경시료에서 기체크로마토그래피-질량분석기를 이용하여 유도체화 과정을 거쳐 7 종의 벤조페논계 자외선차단제의 동시분석법을 개발하였다. 분석된 벤조페논계 자외선차단제는 벤조페논, 벤조하이드롤, 4-하이드록시벤조페논, 2-하이드록시-4-메톡시벤조페논, 2,4-다이하이드록시벤조페논, 2,2‘-다이하이드록시-4-메톡시벤조페논과 2,3,4-트리하이드록시벤조페논이다. 최적조건하에서 수질 (62-114 %)과 토양 (60-125 %)시료는 높은 회수율을 나타내었으며, 낮은 상대표준편차 값을 보여, 분석방법의 정확도와 정밀도를 검증하였다. 검출한계는 분석대상물질에서 5-100 ng/L (또는 kg)의 범위로 산출되었으며,

정량한계는 25-100 ng/L (또는 kg)로 계산되었다. 이와 같이 검증된 분석방법을 통하여 우리나라의 환경시료를 분석한 결과, 수질 (27-204 ppt)보다 토양 (500-18,380 ppt)에서 더 높은 농도로 검출되어, 1 차적으로 수질오염 일어난 이후, 2 차적으로 토양으로 축적 되어지는 것으로 사료된다. 또한, 벤조페논계 자외선차단제의 환경 오염원으로 사료되는 생활하수, 레저용수, 공단배수 중에서 공단배수가 주오염원으로 작용하는 것으로 보여진다.

둘째로, 랫드(rat)의 혈액 중에서 기체크로마토그래피-질량분석기를 이용하여 벤조페존계 자외선차단제의 최적분석방법을 개발하였다. 벤조페논과 2-하이드록시-4-메톡시벤조페논 100 mg kg^{-1} bw 을 랫드에 경구투여하여 시간별로 혈액시료를 채취하였고, 시료는 pH 9.5 에서 에틸아세테이트로 추출하여 유도체화 후 분석하였다. 최적분석조건 하에서, 검량선은 r^2 > 0.999 이상의 좋은 직선성을 보였으며, 76 % 이상의 회수율을 나타내었고, 상대표준편차 9.87 % 이하의 일내 정밀성, 13.89 % 이하의 일간 정밀성과 상대표준오차 -14.59 % 값 이하의 정확성이 검증되었다. 본 분석방법을 적용한 결과, 랫드에 벤조페논과 2-하이드록시-4-메톡시벤조페논을 경구투여한 이후 30 분부터 그 대사체인 벤조하이드롤, 4-하이드록시벤조페논, 2,4-다이하이드로시벤조페논, 2,2‘-다이하이드록시-4-메톡시벤조페논과 2,3,4-트리

하이드록시벤조페논이 검출되었다. 독성동태학적 생체이용률 파라미터는 Win-Nonline 프로그램을 이용하여 산출되었으며, 벤조페논은 8 시간에서 2.06 μg/ml 의 최고농도를 보였고, 2-하이드록시-4-메톡시벤조페논은 3 시간에서 21 ㎍/ml 로 검출되었다. 벤조페논과 2-하이드록시-4-메톡시벤조페논은 다이페닐케톤 구조로서 두개의 방향족 고리를 가지고 있으며, 이러한 구조는 소수성(지방친화성) 분자로 작용하여 상대적으로 빠른 흡수가 일어나는 것으로 사료되어진다.

세번째 연구에서는, 세포수준에서 DNA 손상을 확인하기 위하여 단세포전기영동법(comet assay)을 이용하여 벤조페논계 자외선차단제의 위해성을 평가하였다. 그 결과, 벤조페논과 그 대사체인 4-하이드록시벤조페논은 통계적으로 유의한 DNA 손상을 보였으며 ($P < 0.05$), 벤조하이드롤은 대사활성존재 및 부재하 모두에서 DNA 손상을 유발하지 않는것으로 나타났다. 2-하이드록시-4-메톡시벤조페논은 대사체인 2,4-다이하이드록시벤조페논과 2,3,4-트리하이드록시벤조페논에서 대사활성 존재 및 부재시 모두 DNA 손상을 유발하였으나, 2,2‘-다이하이드록시-4-메톡시 벤조페논은 DNA 손상을 유발하지 않았다. 따라서 본 실험을 통하여 유추한 결과, 벤조페논과 2-하이드록시-4-메톡시벤조페논의

대사체들 중에서 *para*-위치에 하이드록실기를 가진 대사체들에서 DNA 손상을 유발하는 것으로 사료되어진다.

네번째로, 이들 벤조페논계 자외선차단제는 세계야생보호기금과 일본 국립의약품 식품위생연구소에서 내분비계장애물질로 분류되어지고, 다수의 문헌을 통하여 내분비계 호르몬 (특히 성호르몬)과 유사한 작용을 하는 것으로 보고되고 있다. 따라서 본 연구에서는 인간 유방암 세포주인 MCF 7 에 벤조페논계 자외선차단제를 48 시간 동안 처리한 후, cDNA 마이크로어레이 실험을 통해 유전자 발현 양상을 분석하였다. 벤조페논과 4-하이드록시벤조페논, 2-하이드록시-4-메톡시벤조페논에 의해 각각 32 개, 38 개, 30 개 유전자가 유의한 발현 변화를 보였으며, 벤조페논계 자외선차단제에 대해 공통적으로 발현이 증가한 4 개의 유전자와 6 개의 감소한 유전자들을 선별할 수 있었다. 이들 유전자 중에서 발현이 증가한 3 개 유전자와 감소한 3 개의 유전자가 17β-에스트라다이올과 일치하는 결과를 보여 높은 상관관계를 나타내었다. 이들은 세포증식, 갑상선호르몬, 전사조절, 대사작용, 면역작용과 관련된 유전자들로 벤조페논계 자외선차단제의 새로운 지표 유전자로 도출하기 위하여 RT-PCR 을 이용하여 확인시험을 수행하였으며, 추가적으로 다른 벤조페논계 자외선차단제들에 연구가 수반되어야 할 것으로 사료된다.

요약하면, 본 연구에서는 벤조페논계 자외선차단제의 노출에 대하여 각기 다른 독성학적인 기법을 활용하여 다양한 각도에서 접근하고자 하였다. 이러한 연구를 통하여 벤조페논계 자외선차단제의 위해성 평가와 법률 제정에 유용한 기초자료로서 제공되어 질 것으로 사료된다.

핵심되는 말 : 벤조페논, 자외선차단제, 수질, 토양, 가스크로마토그래피-질량분석기, 독성동태학, 유전독성, 단세포전기영동법, 독성유전체학, cDNA 마이트로어레이