Master of Science

Comprehensive Toxicity Evaluation of Bisphenol F via Dermal Exposure

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of the University of Ulsan
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Comprehensive Toxicity Evaluation of Bisphenol F via Dermal Exposure

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Contents

List of Tables

List of Figures

Abstract

Among life chemicals, Bisphenol F(BPF) is used as a substitute for Bisphenol A(BPA) in food containers, water supply pipes, and thermal papers for receipts, and is actually detected in drinking water and human urine samples in Korea. However, due to limited research on the toxicity information on Bisphenol F, it is difficult to prepare appropriate guidelines for use and social anxiety is amplifying. In recent studies, Bisphenol F has also been pointed out as an endocrine disruptor, and related risks have been revealed through research. However, most of these studies focused on human exposure to BPF through direct ingestion or inhalation, and studies evaluating the toxicological effects of BPF mainly through skin contact are lacking. Therefore, in this study, we conducted comprehensive toxicity tests focusing on dermal exposure. Through *in vitro* application, the degree of absorption into the skin and the permeation pattern were evaluated. a single-dose dermal toxicity test, 14-day repeat dermal dose-range finding study, 28-day repeat dermal toxicity test were performed to identify the no observed adverse effect level (NOAEL) and target organs during skin exposure. Skin irritation test and eye irritation test were performed to determine local irritation upon skin exposure, and a skin sensitization test was performed to evaluate delayed hypersensitivity reactions following skin contact.

Results showed that BPF was absorbed through the skin at a high rate, as indicated by the amount of BPF remaining in the epidermis or dermis, compared with other bisphenol analogs. BPF penetrated into the subcutaneous layer at a very fast rate. No toxicological changes or local irritation were observed after skin exposure to BPF. However, BPF induced a strong sensitization similar to that of positive (HCA). the findings of this study demonstrated the significant implications of skin exposure through the results of high rates of skin penetration and skin absorption of BPF, and the result of extensive toxicological evaluation of skin exposure to BPF confirmed BPF inducing skin sensitization.

Introduction

Bisphenol A(BPA, 4,4′-(propane-2,2-diyl)diphenol), an industrial chemical used worldwide, has high strength, heat resistance, and transparency. BPA is used in synthesis of polycarbonate plastic, synthesis of epoxy resin, phenoplast resin, unsaturated polyester resin, flame retardants, antioxidant in thermal paper, and it is a material with a high potential for human exposure. In the early 1960s, the Food and Drug Administration (FDA) approved the use of BPA-containing polycarbonate and epoxy resins in food storage containers and packaging (Lim et al. 2009; Tsai 2006). The global market for Bisphenol A is estimated to be more than 6.2 million tons in the year 2020, growing at a compound annual growth rate(CAGR) of 2% over the analysis period 2020-2027. Led by countries such as Australia, India, and South Korea, the market in Asia-Pacific is forecast to reach 960.2 thousand tons by the year 2027 (Newswire, 2021).

Despite the widespread use of BPA, the safety of bisphenol A has been a constant issue since the 1990s, and many studies have been conducted accordingly. The female hormone-like action of BPA was identified, and as an endocrine disrupting substance, it was reported to cause decreased fertility and developmental disorders, metabolic disorders, hypertension, and premature puberty (Bae et al. 2012; Hwang et al. 2018; Ikezuki et al. 2002; Inadera et al. 2015; Rochester 2013; Vandenberg et al. 2007; Weber et al. 2015). In addition, androgen-like action and decreased fertility in male experimental animals were identified, and it was reported that it has a very high sensitivity, especially in infants and young children (De Campos et al. 2019; Manfo et al. 2014; Pollard et al. 2019; Zhou et al. 2020). Given these growing concerns, chemicals structurally similar to BPA, particularly Bisphenol F (BPF, 4,4′-dihydroxydiphenyl-methane) and Bisphenol S (BPS, 4,4′-sulfonylbisphenol), are gradually replacing its use in various industries (Rocha et al. 2015; Vervliet et al. 2019).

In contrast to BPA, when used in packaging, BPF does not leak bisphenol from the coated inner wall into food and beverages, making it is a primary next-generation candidate for replacing BPA. Currently, it has replaced BPA in products such as the internal coatings of food, pharmaceutical, and cosmetic containers, thermal paper for receipts, and the coatings of water pipes (Nakiwala et al. 2020; Office of Waterworks, Seoul Metropolitan Government 2014). Recently, as major advanced countries such as the United States and Europe accelerated their policies to expand eco-friendly energy, the demand for BPA, which is used as a material for lightening automobiles and a polycarbonate material for 'Blades (wings)' of wind power generation facilities, is rapidly increasing. Accordingly, the demand for BPF as an alternative BPA is expected to increase significantly.

Because BPF and BPA have similar structures, many studies have assessed its potential as an endocrine disrupter. Moreover, several studies have shown that BPF can cause hormonal effects similar to those of BPA. BPF is confirmed to have an endocrine-disrupting effect causing an increase in tyrosin hormone (T3/T4) in zebra-fish (Lee et al. 2019). It has been reported that it affects estrogen activity and causes sperm production inhibition and testosterone production decrease, and is evaluated as an endocrine disruptor at a similar level to BPA (Ullah et al. 2018; Rochester and Bolden 2015; Chen et al. 2016). In addition, it has been proven through prior studies that it induces inflammation related to oxidative stress (Huang et al. 2020; Liang et al. 2020; Xie et al. 2020) and induces hepatotoxicity in a 28-day repeated administration oral toxicity test using rats (Higashihara et al. 2007), leading the U.S. Environmental Protection Agency to classify it as a harmful substance (U.S. Environmental Protection Agency, 2015).

However, although previous studies have predominately focused on human exposure to BPF via direct consumption or inhalation (Eladak et al., 2015; Dekant and Völkel 2008; Lakind and Naiman 2011), recent studies have indicated that bisphenol analogs remain in the body much longer when absorbed into the skin through touch, compared to when ingesting them

through food or beverages (Liu and Martin, 2017). However, *in vivo* toxicity studies examining the skin exposure route are still lacking, and the amount of dermally absorbed bisphenol that remains in the body, as well as its subsequent effects, have not been clearly established. Therefore, studies investigating the toxicological effects associated with skin contact, among various other BPF exposure routes, are needed. The amount of dermally absorbed bisphenol that remains in the body following exposure and subsequent effects remain to be determined. Therefore, the aim of this study was to examine the subsequent toxicological effects associated following skin contact, and compared to various other BPF exposure routes.

A hazard assessment to humans is possible through comprehensive toxicity tests in dermal route. Acute dermal toxicity provides information on health hazards likely to arise from a short-term exposure by the dermal route. This data may serve as an initial step in establishing a dosage regimen in subchronic studies. The twenty-eight day repeated dermal toxicity test provides information on the possible health hazards likely to arise from repeated exposure. This study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation, and can provide an estimate of a NOAEL (no observed adverse effect level) which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. The purpose of the skin irritation and eye irritation test is to evaluate the skin/eye irritation that occurs after BPF is directly applied to the skin/eyes of animals. The skin sensitization test evaluates delayed hypersensitivity reactions to determine whether BPF induces contact-mediated allergy.

In the current study, we conducted comprehensive toxicity tests focusing on dermal exposure. we assessed the extent to which BPF penetrates and remains in the skin using the Franz method (Franz, 1975), also investigating the effects of skin exposure *in vivo*. Furthermore, a single-dose dermal toxicity test, 14-day repeat dermal dose-range finding study, 28-day repeat dermal toxicity test were performed to identify the no

observed adverse effect level (NOAEL) and target organs during skin exposure. Skin irritation test and eye irritation test were performed to determine local irritation upon skin exposure, and a skin sensitization test was performed to evaluate delayed hypersensitivity reactions following skin contact.

Materials and methods

1. Test chemicals

The BPF (ZS20190510, purity: 99.1%) used in this study was purchased from Orient Chemical Enterprises. Dimethyl sulfoxide (2018I5036, CAS: 67-68-5) and corn oil (MKCH1635, Sigma-Aldrich, Inc.) were used as vehicle control substances. Benzoic acid (MKCG6487, CAS: 65-85-0) and α-hexylcinnamaldehyde (MKCF6971, CAS: 101-86-0) were employed as positive control substances in the skin absorption and skin sensitization tests, respectively.

2. Chemical Characterization

BPF concentration was determined using high-performance liquid chromatography (HPLC). The HPLC diode array detection system (Infinity 1260 series, Agilent Technologies, USA) with a Zorbax Eclipse Plus C18 column (3.5 μm, 4.6 × 150 mm) was utilized for analysis. HPLC analysis conditions for samples obtained using Franz diffusion cells are summarized in Table 1. The homogeneity test of the upper layer, the middle layer, and the lower layer, and the stability of the test substance preparation day (day 0), 4 hours, 1 day, 3 days, and 7 days after preparation, were confirmed using this test.

Table 1. High-performance liquid chromatography(HPLC) analysis conditions.

Parameters	Bisphenol F	Benzoic acid
Oven Temp.	40 °C	40 °C
Mobile phase (isocratic)	Water/acetonitrile = 50:50 (v/v)	Water/acetonitrile = 60:40 (v/v)
Flow rate	1 mL/min	1 mL/min
Wavelength	250 nm	250 nm
Injection vol.	10 μL	10 μL
Retention time	2.2 min	2.3 min
Detector	Diode array detector	

3. Skin absorption test: in vitro using the Franz method

The test was performed in accordance with Organization for Economic Cooperation and Development (OECD) guidelines (2004). A receptor volume of 5 ml was used in the Franz (1975) diffusion cell (LOGAN Instruments, USA) in the dermal absorption test(Champmartin et al. 2020; Toner et al. 2018). Human full skin (Batch No. DR161217-05, DermaLab) was placed between the donor and receptor chambers with the stratum corneum facing upward. The receptor chamber was then filled with receptor fluid of ethanol/water solution (5 ml, 1:1). The temperature and humidity of the absorption device and skin were maintained at 32 °C and 30–70%, respectively. The skin area used was 0.64 cm^2; 100 μl (6 mg, 6 %) of BPF was applied onto the skin for a total of 4 times. After 1, 2, 4, 5, 6 or 24 hr, 5 ml BPF that had permeated into the receptor chamber was collected and replaced with an equal volume (5 ml) of receptor fluid. At the end of the experiment, the surface of the skin and donor chamber were washed with ethanol/water (1:1, v/v) to collect any residual BPF. Chemical on the skin was collected only from the area to which the test substance was applied, and this was achieved by soaking the skin in 10 ml ethanol/water (1:1, v/v), followed by sonication (1 hr) and elution (24 hr). The sample was then centrifuged (3,000 rpm, 15 min. 20 °C), and supernatant collected. The extracted sample was stored at −80 °C until further HPLC analysis.

Samples collected within 24 hr were quantified on a graph plotting permeation amount by time. The total absorption rate (skin absorption amount + skin permeation amount) was calculated by measuring the absorption rate (applied dose = washed amount + skin absorption amount + skin permeation amount). Flux (μg/hr·cm^2), the amount of material that permeates a certain area per unit of time, was calculated. The flux value was divided by initial concentration (g/cm^2) of BPF to calculate the transmission coefficient (Kp, cm/hr) (Reichling et al. 2006).

4. Acute dermal toxicity test

This test was conducted to determine toxicity symptoms and approximate lethal dose (ALD) of a single transdermal administration of BPF. A total of 40 Sprague–Dawley rats (Orientbio Inc., 7–8 weeks Crl:CD) were used, 20 male and 20 female, with 5 males and females in each group. Test groups were administered in mg/kg doses of BPF 160 (low), 400 (intermediate), or 1,000 (high). Test group results were compared with those of vehicle control group. Dead animals, clinical signs, weight changes, and autopsy findings of surviving animals were measured during the experiment. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC; IA19-01727) of Korea Conformity Laboratories (KCL).

5. Twenty-eight day repeated dermal toxicity test

Following repeated transdermal administration of BPF for 4 weeks, a 28-day repeated toxicity test was conducted to determine the no-observed-adverse-effect-level (NOAEL) in rats. The test method was performed in accordance with OECD guidelines (1981).

As a result of conducting a 2-weeks repeated dermal dose range finding(DRF) study, the female 1,000 mg/kg/day/day group showed a low weight gain (about 10% weight loss compared to the vehicle control group). Therefore, in the 4-weeks repeated dermal test, the maximal feasible dose(MFD) of 1,000 mg/kg/day, which is the same dose as in the DRF study, was set as the highest dose group. With the highest dose of 1,000, the common ratio was set at about 3, and 4 dose groups(10, 30, 100, 300) were added. Therefore, a total of 6 groups were set, including the vehicle control group administered only with corn oil.

A total of 60 Sprague–Dawley rats (Orientbio Inc., Crl:CD, 7–8 weeks) were used, 30 male and 30 female, with 5 males and females in each group. Treatment groups were compared with vehicle controls. During the experiment, mortality, general symptoms, weight changes, and food intake were recorded. An eye test, urine chemistries, urine sediment test, urine volume, hematological chemistries, blood clotting time test, blood

biochemical parameters organ weights, and gross and histopathological examinations at autopsy were performed. All experiments were approved by the IACUC (IA19-02233) of KCL.

6. Skin and eye irritation tests

These tests were performed in accordance with OECD guidelines (OECD 2002, 2012). To assess skin irritation, 0.5 g BPF was applied to the abraded and non-abraded skin of 6 male New Zealand white rabbits (PiZhou Dongfang Rabbit Breeding Co., Ltd) for 24 hr. Skin reactions including erythema, crust formation, and edema were observed 24 and 72 hr after administration. The degree of skin irritation is calculated according to the calculation method (total skin reaction score/4) of the Primary Irritation Index (P.I.I.) by Draize method (Draize, Woodard, and Calvery 1994).

In order to assess irrigation of the eye mucosa, 0.1 g BPF was administered once to the conjunctival sac of 9 male New Zealand white rabbits (PiZhou Dongfang Rabbit Breeding Co., Ltd). Three animals in the eye wash group were washed with sterile physiological saline for 1 min, 20–30 sec after administration, and 6 animals in the non-wash group were not washed. Eye irritation including corneal opacity, iris reaction, conjunctival redness, edema, and discharge was noted on days 1, 2, 3, 4, and 7 after BPF administration. All experiments were approved by the IACUC (IA20-00407, IA20-00408) of KCL

7. Skin sensitization test

This test was performed in accordance with OECD guidelines (Magnusson and Kligman 1969; OECD 1992). To evaluate skin sensitization to BPF, the guinea pig maximization test was performed using 20 female guinea pigs (Koatech Inc., 4 weeks old, HlaKoat:Hartley). Corn oil was used as the vehicle at a concentration of 20% (w/v) for intradermal and topical inductions. The intradermal induction (Day 0) was performed simultaneously in the upper, middle, and lower parts via 0.1 ml intradermal injections of Freund's Complete Adjuvant (Sigma-Aldrich Korea Ltd.) at each site. Sodium dodecyl sulfate (10%) was used to induce non-invasive local stimulation. The topical induction (Day 7) was performed by applying

0.2 ml BPF solution to the shoulder region. This was then covered with non-irritating vinyl (Tegaderm 1624W, 3M) and bandaged with non-irritating tape (Micropore 1530-1, 3M) for 48 hr. Challenge exposure (Day 21) was conducted in all animals. Briefly, 0.1 ml either BPF or vehicle was completely loaded into each patch. The test substance and vehicle were applied to the left and right flank, respectively, of the animal using non-irritating adhesive vinyl. The site of application on each animal was wrapped in a bandage for 24 hr.

Skin reactions were assessed using Magnusson and Kligman (1969) skin reaction evaluation criteria where the affected area was observed 24 and 48 hr after removing the inducing patch. The sensitization rate was calculated for skin sensitization evaluation.

$$* \text{ Sensitization rate (\%)} = \frac{\text{No. of animals with positive reaction}}{\text{No. of tested animals}} \times 100$$

All experiments were approved by the IACUC (IA20-00407, IA20-00240) of KCL.

Table 2. Magnusson and Kligman criteria of skin sensitization.

sensitization rate (%)	Grade	Class
0～8	I	Weak
9～28	II	Mild
29～64	III	Moderate
65～80	IV	Strong
81～100	V	Extreme

8. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 12.0 software (IBM, Chicago, IL, U.S.A.). The criterion for statistically significant was set at $p < 0.05$. The incidence rates were expressed as % values.

Results

1. Analytical method validation for BISPHENOL F by LC-MS/MS

The calibration curve that was plotted using Bisphenol F standard solution (5.000, 10.000, 20.000 and 40.000 mg/L) by LC-MS/MS was linear. And the regression coefficient (R^2) of the calibration curve was 0.9995(Figure 1). Bisphenol F standard solutions of 5.000 and 40.000 mg/L were measured seven times respectively. At the results, a mean of a relative error (% Error) was -5.8～4.6 %, -1.3～0.4 % and it was less than ±10 %, the permitted standard of accuracy. Bisphenol F standard solutions of 5.000 and 40.000 mg/L were measured seven times respectively. At the results, a mean of CV(coefficient of variation) was 0.4 % and 0.7%, respectively and they were less than 5 %, the permitted standard of precision. As the result of the quantitative analysis of BPF, LOD (Limit of Detection) was 0.065 mg/L and LOQ (Limit of Quantitation) was 0.196 mg/L.

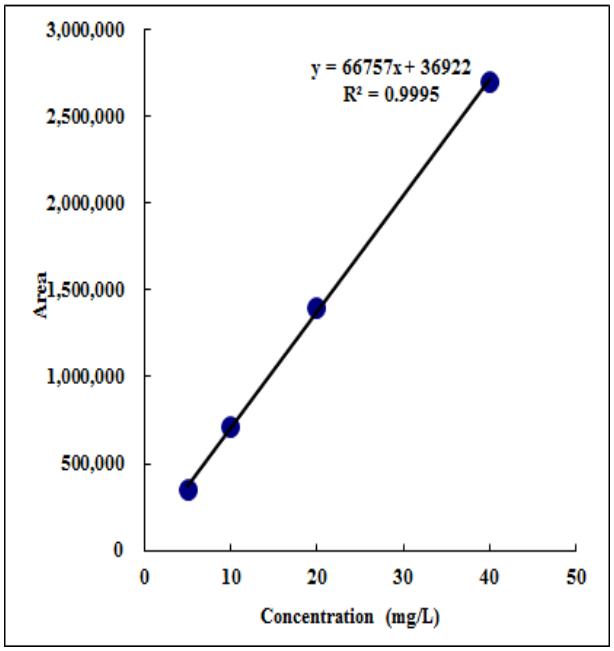

Figure 1. Calibration curve of Bisphenol F standard solution in analysis method review.

2. Skin absorption test: in vitro using the Franz method

In this study, a Franz diffusion cell was used according to the Franz method (1975) to assess skin absorption and permeation characteristics of BPF over time. The degree of skin penetration/hr was determined for BPF and benzoic acid with the recovery rate of each found to be 77.8% and 83.4%. In the case of BPF, the amount of substance remaining in the skin was 0.11 mg (1.7%), the amount of substance actually penetrated through the skin and absorbed under the dermis was 1.64 mg (26.5%), and the amount of test substance lost regardless of absorption was 3.08 mg (49.6%), respectively. In the case of Benzoic acid, remaining amount in the skin was 0.11 mg (1.7%), skin penetrated amount was 5.13 mg (80.9%), and unabsorbed amount was 0.04 mg (0.7%), respectively (Figure 2 and Table 4).

Figure 2. Comparative analysis of absorption by skin area.
The amount of absorption was compared for the three skin area between BPF and Benzoic acid. 1.The amount of BPF/Benzoic acid remaining in the skin, 2.The amount of BPF/Benzoic acid that actually penetrates the skin absorbed under the dermis, 3.The amount of the BPF/Benzoic acid lost regardless of absorption.

Absorption rates were measured for each time period (0h, 1h, 2h, 3h, 4h, 5h, 6h, 24h), and in the case of BPF, the average cumulative absorption was 0.01 mg, 0.05 mg, 0.13 mg, 0.25 mg, 0.41 mg, 0.55 mg, 1.64 mg, and benzoic acid was measured to be 0.22 mg, 0.79 mg, 1.49 mg, 2.16 mg, 2.86 mg, 3.52 mg, and 5.13 mg. Therefore, it was confirmed that the penetration rate increased as the time increased from 0 hr to 24 hrs. (Figure 3 and Table 3).

Figure 3. Cumulative penetration rate per hour for bisphenol F and benzoic acid.

As a result of analyzing the penetration rate (%, Cumulative absorption amount/Administration dose × 100) for each amount of absorption in a graph, it was confirmed that it gradually increased as time passed

The mean dermal delivery (skin + absorbed dose), mean residual amount in the skin, and mean absorbed dose (receptor fluid + receptor chamber wash) of BPF were 28.2% (absorption amount 1.75 mg), 1.7% (absorption amount 0.11 mg), and 26.5% (absorption amount 1.64 mg) and in case of benzoic acid were 82.6% (absorption amount 5.24 mg), 1.7% (absorption amount 0.11 mg), and 80.9% (absorption amount 5.13 mg), respectively. Flux (μg/h/cm²) and permeability coefficient (Kp, cm/hr) were calculated based upon the measured data using Marzulli et al (1969) Kp formula. These calculations revealed that the penetration speed of benzoic acid and BPF were "very fast"(Kp : 1.3E-01) and "fast"(Kp : 2.2E-02) respectively (Figure 4 and Table 5).

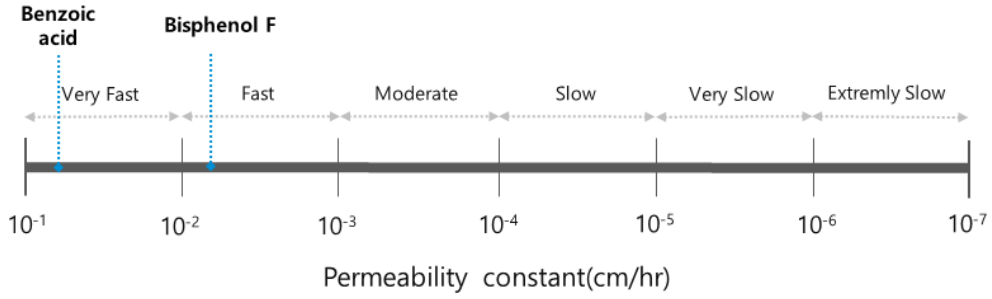

Figure 4. Evaluation of penetration degree between Bisphenol F and Benzoic acid.

Analysis of the permeability of bisphenol F according to Marzulli fomula (Marzulli, 1969). As a result, the BPF permeability constant(Kp) was 2.2 x 10^{-2} and the benzoic acid Kp was 1.3 x 10^{-1}. Therefore, BPF and benzoic acid showed fast and very fast permeability, respectively.

Table 3. Amount of skin-permeated of bisphenol F and benzoic acid.

Bisphenol F (mg)											
	1 h	2 h	3 h	4 h	5 h	6 h	24 h	Washing	Skin	Total	Recovery
1	0.01	0.04	0.11	0.23	0.40	0.57	1.01	3.58	0.06	4.66	75.0
2	0.01	0.04	0.11	0.22	0.34	0.40	0.59	3.78	0.13	4.50	72.5
3	0.01	0.04	0.09	0.18	0.29	0.41	3.20	1.95	0.12	5.27	85.0
4	0.02	0.08	0.21	0.38	0.62	0.83	1.76	3.00	0.12	4.88	78.7
Mean	0.01	0.05	0.13	0.25	0.41	0.55	1.64	3.08	0.11	4.83	77.8
SD	0.01	0.02	0.05	0.09	0.14	0.20	1.15	0.82	0.03	0.34	5.4
Benzoic acid (mg)											
	1 h	2 h	3 h	4 h	5 h	6 h	24 h	Washing	Skin	Total	Recovery
1	0.26	1.01	1.91	2.74	3.52	4.16	5.22	0.02	0.14	5.39	85.0
2	0.21	0.79	1.52	2.25	3.01	3.73	5.22	0.04	0.08	5.34	84.3
3	0.26	0.82	1.49	2.14	2.85	3.55	5.20	0.03	0.12	5.35	84.4
4	0.14	0.54	1.02	1.51	2.05	2.62	4.87	0.08	0.11	5.06	79.8
Mean	0.22	0.79	1.49	2.16	2.86	3.52	5.13	0.04	0.11	5.28	83.4
SD	0.05	0.20	0.36	0.51	0.61	0.65	0.17	0.03	0.03	0.15	2.4

SD, standard deviation.

Table 4. Absorption profile of bisphenol F and benzoic acid.

	Absorption amount (mg)		% Applied dose	
	Bisphenol F	Benzoic acid	Bisphenol F	Benzoic acid
Skin	0.11	0.11	1.7	1.7
Absorbed dose	1.64	5.13	26.5	80.9
Dermal delivery[a]	1.75	5.24	28.2	82.6
Washing[b]	3.08	0.04	49.6	0.7
Total	4.83	5.28	77.8	83.4

a) Dermal delivery = Skin + Absorbed dose;

b) Washing = Skin washings + Cell washing; SD, standard deviation

Table 5. Permeability rates and coefficients of test substance following the exposure of human full skin.

	Bisphenol F	Benzoic acid
Flux (μg/h/cm²)	136.7 ± 101.2	805.0 ± 405.2
Kp (cm/h)	2.2E-02	1.3E-01

3. Acute dermal toxicity test

Toxicity symptoms and approximate lethal dose (ALD) of Bisphenol F by single transdermal administration are investigated. Using male and female Sprague-Dawley (SD) rats, 160 (low-dose group), 400 (medium-dose group), and 1,000 (high-dose group) doses of mg/kg were set in the test group and compared with the excipient control group, and the duration of the experiment During the period, the occurrence of dead animals, general symptoms, weight changes, and autopsy findings of surviving animals at the end of the experiment were observed. In the control group, 160, 400, and 1,000 mg/kg groups, the body weights of 3 males and 5 females on the 1st day after administration of the test substance decreased compared to before administration. As a result of autopsy at the end of the experiment, no specific macroscopic findings were observed in all test animals(Figure 5).

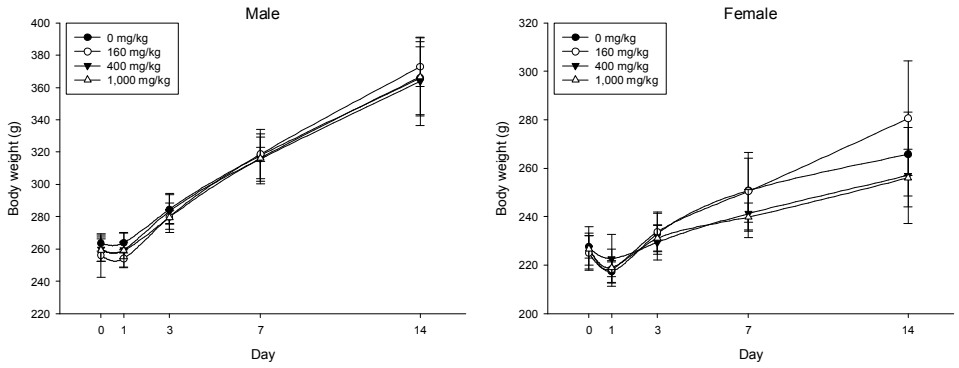

Figure 5. Body weight of male and female rats in acute dermal toxicity test.
As a result of weight measurement, on the 1st day after administration of BPF, a decrease in average body weight was observed in both male and female. However, it is not the effect of the BPF because the degree is mild and a weight change with a similar tendency was observed in the vehicle control group.

4. Twenty-eight day repeated dermal toxicity test

No apparent toxicity symptoms were observed following the single transdermal administration of BPF to Sprague–Dawley rats, whereas the approximate lethal dose (ALD) was found to exceed 1,000 mg/kg/day in both males and females. A two-weeks preliminary DRF study was conducted based upon the ALD. In the same manner as the preliminary test, the maximal feasible dose (MFD) of 1,000 mg/kg/day was set as the highest dose. Four additional dose groups were established, and repeated administration for 4 weeks performed.

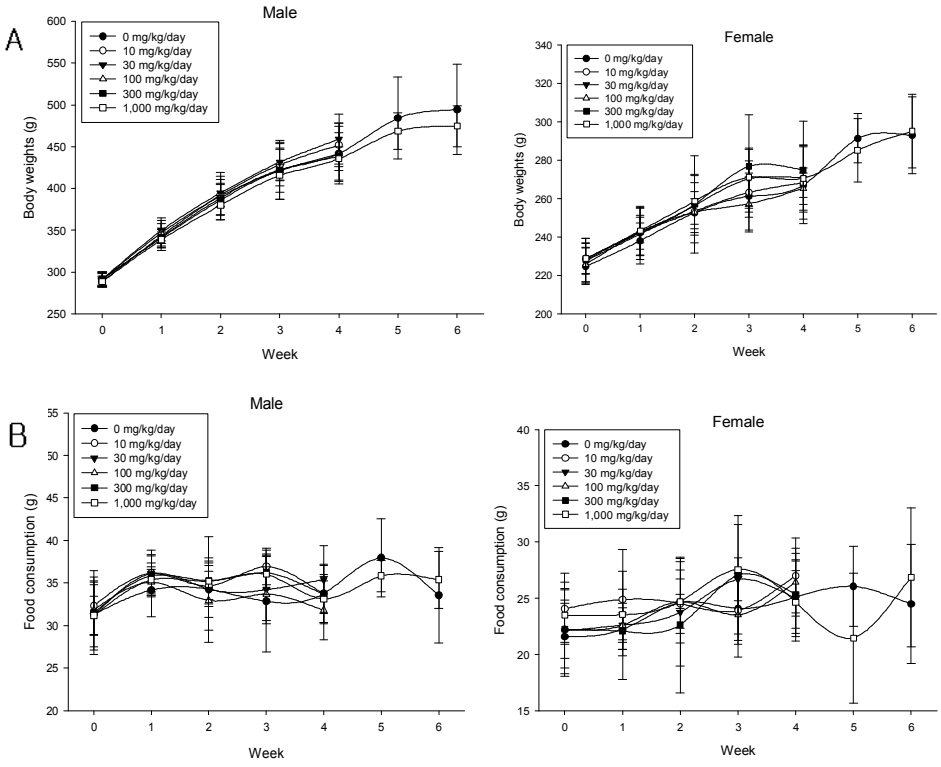

Figure 6. Body weight and food consumption of male and female rats in 28-day repeated dermal toxicity test.

A) Body weight change during the experiment period of male and female rats. There was no significant body weight change during the whole study period. B) Food consumption changes during the whole study period. There was no significant food consumption changes during the whole study period.

The 28-day repeated dermal toxicity test showed no abnormal findings in body weight, food intake, eye tests, urine tests, urine sedimentation, urine amount, or blood clotting time in all male and female rat treatment groups for the duration of the experiment (Figure 6). Hematological testing demonstrated increased white blood cells and neutrophil count in the male treatment group receiving 30 mg/kg/day BPF. The basophil % rose in the 100 mg/kg/day group (Table 6 and 7). A dose–response correlation (Greaves 2007) was not observed even though increased number of white blood cells and neutrophils, and basophil % levels was recorded in all male treatment groups; thus, such elevated levels are not considered to be categorically attributed to BPF.

Further, hematochemical testing showed increased total bilirubin and sodium in the male 1,000 mg/kg/day treatment group. In female rats, total bilirubin was elevated in the 100 and 1,000 mg/kg/day treatment groups. Total cholesterol and albumin were increased in the 30 mg/kg/day treatment group, whereas sodium rose in both 300 and 1,000 mg/kg/day treatment groups (Table 8 and 9). Although total bilirubin, total cholesterol, albumin, and sodium levels in the female treatment groups was elevated, the observed changes were minimal and within the range of historical background data.

Table 6. Hematological values of male rats in 28-day repeated dermal toxicity test.

WEEK	GROUP(㎎/㎏/day)																							
	G1(0)				G2(10)				G3(30)				G4(100)				G5(300)				G6(1,000)			
WBC[1](K/μL)	5.58	±	1.19	(5)	6.15	±	2.61	(5)	9.45*	±	1.90	(5)	7.81	±	1.71	(5)	7.83	±	1.87	(5)	6.40	±	0.90	(5)
NE[2](K/μL)	0.89	±	0.28	(5)	0.87	±	0.27	(5)	1.62*	±	0.38	(5)	1.24	±	0.39	(5)	1.05	±	0.35	(5)	1.11	±	0.14	(5)
EO[3](K/μL)	0.08	±	0.01	(5)	0.09	±	0.03	(5)	0.12	±	0.03	(5)	0.10	±	0.03	(5)	0.08	±	0.02	(5)	0.08	±	0.01	(5)
BA[4](K/μL)	0.00	±	0.00	(5)	0.00	±	0.00	(5)	0.00	±	0.01	(5)	0.00	±	0.01	(5)	0.00	±	0.00	(5)	0.00	±	0.00	(5)
LY[5](K/μL)	4.45	±	1.11	(5)	4.99	±	2.34	(5)	7.41	±	1.81	(5)	6.25	±	1.41	(5)	6.53	±	1.62	(5)	5.04	±	0.91	(5)
MO[6](K/μL)	0.14	±	0.06	(5)	0.15	±	0.08	(5)	0.26	±	0.13	(5)	0.18	±	0.06	(5)	0.14	±	0.06	(5)	0.14	±	0.03	(5)
LUC[7](K/μL)	0.02	±	0.03	(5)	0.05	±	0.05	(5)	0.04	±	0.03	(5)	0.04	±	0.04	(5)	0.03	±	0.03	(5)	0.03	±	0.02	(5)
NEP[8](%)	16.3	±	4.6	(5)	15.5	±	5.3	(5)	17.8	±	5.6	(5)	15.9	±	4.5	(5)	13.6	±	3.9	(5)	17.5	±	3.2	(5)
EOP[9](%)	1.4	±	0.2	(5)	1.6	±	0.7	(5)	1.3	±	0.2	(5)	1.4	±	0.6	(5)	1.0	±	0.2	(5)	1.3	±	0.2	(5)
BAP[10](%)	0.0	±	0.0	(5)	0.0	±	0.1	(5)	0.0	±	0.1	(5)	0.1*	±	0.0	(5)	0.0	±	0.0	(5)	0.0	±	0.0	(5)
LYP[11](%)	79.5	±	5.0	(5)	79.7	±	5.6	(5)	77.9	±	4.6	(5)	79.9	±	4.7	(5)	83.1	±	4.0	(5)	78.4	±	3.9	(5)
MOP[12((%)	2.4	±	0.9	(5)	2.4	±	0.6	(5)	2.6	±	0.9	(5)	2.2	±	0.5	(5)	1.8	±	0.6	(5)	2.3	±	0.7	(5)
LUP[13](%)	0.4	±	0.4	(5)	0.7	±	0.5	(5)	0.4	±	0.2	(5)	0.6	±	0.4	(5)	0.4	±	0.3	(5)	0.5	±	0.3	(5)
RBC[14](M/μL)	7.96	±	0.15	(5)	7.78	±	0.28	(5)	7.90	±	0.12	(5)	8.10	±	0.17	(5)	8.12	±	0.26	(5)	8.08	±	0.18	(5)
Hb[15](g/dℓ)	16.4	±	0.4	(5)	16.0	±	0.7	(5)	16.7	±	0.6	(5)	16.7	±	0.4	(5)	16.7	±	0.4	(5)	16.9	±	0.7	(5)
RDW[16](%)	10.5	±	0.2	(5)	11.0	±	0.6	(5)	11.0	±	0.5	(5)	10.4	±	0.3	(5)	10.8	±	0.4	(5)	10.7	±	0.3	(5)
HCT[17](%)	45.5	±	0.9	(5)	44.1	±	1.6	(5)	45.6	±	1.3	(5)	46.4	±	1.0	(5)	45.9	±	1.2	(5)	46.2	±	1.9	(5)
MCV[18](fL)	57.2	±	0.4	(5)	56.7	±	1.3	(5)	57.7	±	0.9	(5)	57.3	±	1.3	(5)	56.6	±	1.3	(5)	57.2	±	2.2	(5)
MCH[19](pg)	20.6	±	0.3	(5)	20.5	±	0.6	(5)	21.2	±	0.4	(5)	20.7	±	0.5	(5)	20.6	±	0.4	(5)	21.0	±	1.0	(5)
MCHC[20](g/dℓ)	36.1	±	0.6	(5)	36.2	±	0.4	(5)	36.7	±	0.6	(5)	36.1	±	0.2	(5)	36.4	±	0.2	(5)	36.6	±	0.7	(5)
Reti[21](%)	1.34	±	0.74	(5)	1.56	±	0.97	(5)	1.96	±	0.65	(5)	1.42	±	0.78	(5)	2.30	±	0.17	(5)	2.36	±	0.19	(5)
MetHb[22](%)	2.1	±	0.2	(5)	2.5	±	0.6	(5)	2.3	±	0.5	(5)	2.2	±	0.2	(5)	2.0	±	0.2	(5)	2.2	±	0.2	(5)
PLT[23](K/μL)	1187	±	143	(5)	1111	±	130	(5)	1092	±	122	(5)	1184	±	90	(5)	1116	±	118	(5)	1032	±	86	(5)
MPV[24](fL)	6.7	±	0.3	(5)	6.7	±	0.2	(5)	6.7	±	0.2	(5)	6.8	±	0.2	(5)	6.8	±	0.5	(5)	6.8	±	0.5	(5)

Mean±S.D (Number of animals)

1: White blood cell, 2: Neutrophil, 3: Eosinophil, 4: Basophil, 5: Lymphocyte, 6: Monocyte, 7: Large unstained cell, 8: Percent of neutrophil, 9: Percent of eosinophil, 10: Percent of basophil, 11: Percent of lymphocyte, 12: Percent of monocyte, 13: Percent of large unstained cell, 14: Red blood cell, 15: Hemoglobin, 16: Red cell distribution width, 17: Hematocrit, 18: Mean corpuscular volume, 19: Mean corpuscular hemoglobin, 20: Mean corpuscular hemoglobin concentration, 21: Reticulocyte, 22: Methemoglobin, 23: Platelet, 24: Mean platelet volume *: Significant difference compared with control group value, $p<0.05$

Table 7. Hematological values of female rats in 28-day repeated dermal toxicity test.

WEEK	GROUP(㎎/㎏/day)																	
	G1(0)			G2(10)			G3(30)			G4(100)			G5(300)			G6(1,000)		
WBC[1](K/μL)	5.97	± 1.29	(5)	6.40	± 0.83	(5)	5.94	± 1.34	(5)	7.27	± 1.92	(5)	6.25	± 1.30	(5)	5.89	± 1.07	(5)
NE[2](K/μL)	0.61	± 0.20	(5)	0.72	± 0.28	(5)	0.57	± 0.29	(5)	0.74	± 0.37	(5)	0.51	± 0.13	(5)	0.55	± 0.23	(5)
EO[3](K/μL)	0.09	± 0.02	(5)	0.09	± 0.02	(5)	0.07	± 0.02	(5)	0.07	± 0.01	(5)	0.07	± 0.02	(5)	0.08	± 0.02	(5)
BA[4](K/μL)	0.00	± 0.00	(5)	0.00	± 0.00	(5)	0.00	± 0.00	(5)	0.00	± 0.00	(5)	0.00	± 0.00	(5)	0.00	± 0.00	(5)
LY[5](K/μL)	5.06	± 1.24	(5)	5.45	± 0.73	(5)	5.09	± 1.00	(5)	6.23	± 1.86	(5)	5.49	± 1.11	(5)	5.07	± 0.93	(5)
MO[6](K/μL)	0.15	± 0.08	(5)	0.10	± 0.04	(5)	0.15	± 0.04	(5)	0.14	± 0.03	(5)	0.12	± 0.07	(5)	0.14	± 0.04	(5)
LUC[7](K/μL)	0.06	± 0.03	(5)	0.04	± 0.02	(5)	0.06	± 0.03	(5)	0.08	± 0.03	(5)	0.05	± 0.02	(5)	0.06	± 0.03	(5)
NEP[8](%)	10.4	± 3.7	(5)	11.2	± 3.9	(5)	9.1	± 2.9	(5)	10.4	± 5.3	(5)	8.2	± 0.9	(5)	9.3	± 3.2	(5)
EOP[9](%)	1.7	± 0.5	(5)	1.4	± 0.4	(5)	1.2	± 0.4	(5)	1.0	± 0.2	(5)	1.3	± 0.5	(5)	1.3	± 0.4	(5)
BAP[10](%)	0.0	± 0.0	(5)	0.0	± 0.0	(5)	0.1	± 0.1	(5)	0.0	± 0.1	(5)	0.1	± 0.0	(5)	0.0	± 0.0	(5)
LYP[11](%)	84.4	± 4.5	(5)	85.1	± 3.9	(5)	86.2	± 2.9	(5)	85.4	± 5.7	(5)	87.9	± 1.7	(5)	86.0	± 3.9	(5)
MOP[12]((%)	2.7	± 1.2	(5)	1.5	± 0.4	(5)	2.5	± 0.3	(5)	2.1	± 0.7	(5)	1.8	± 0.8	(5)	2.4	± 0.7	(5)
LUP[13](%)	1.0	± 0.5	(5)	0.7	± 0.4	(5)	0.9	± 0.3	(5)	1.1	± 0.4	(5)	0.9	± 0.1	(5)	1.0	± 0.3	(5)
RBC[14](M/μL)	7.90	± 0.25	(5)	7.78	± 0.50	(5)	7.80	± 0.36	(5)	7.51	± 0.27	(5)	7.71	± 0.12	(5)	7.65	± 0.29	(5)
Hb[15](g/dℓ)	15.5	± 0.4	(5)	15.4	± 0.5	(5)	15.3	± 1.0	(5)	15.2	± 0.3	(5)	15.7	± 0.2	(5)	15.6	± 0.6	(5)
RDW[16](%)	10.7	± 0.4	(5)	10.8	± 0.3	(5)	10.6	± 0.4	(5)	10.4	± 0.2	(5)	10.4	± 0.3	(5)	10.6	± 0.4	(5)
HCT[17](%)	42.0	± 1.3	(5)	42.7	± 1.7	(5)	42.2	± 2.9	(5)	41.2	± 1.1	(5)	43.0	± 1.2	(5)	42.6	± 1.6	(5)
MCV[18](fL)	53.2	± 0.9	(5)	55.0	± 1.8	(5)	54.0	± 1.8	(5)	54.9	± 0.8	(5)	55.8	± 1.8	(5)	55.7	± 1.5	(5)
MCH[19](pg)	19.6	± 0.3	(5)	19.8	± 0.8	(5)	19.5	± 0.7	(5)	20.2	± 0.5	(5)	20.4	± 0.4	(5)	20.4	± 0.7	(5)
MCHC[20](g/dℓ)	36.8	± 0.5	(5)	35.9	± 0.4	(5)	36.2	± 0.5	(5)	36.8	± 0.6	(5)	36.5	± 0.6	(5)	36.6	± 0.4	(5)
Reti[21](%)	1.00	± 0.46	(5)	1.12	± 0.89	(5)	1.32	± 0.73	(5)	0.60	± 0.20	(5)	0.66	± 0.23	(5)	1.52	± 1.08	(5)
MetHb[22](%)	2.3	± 0.6	(5)	2.3	± 0.4	(5)	2.2	± 0.3	(5)	2.4	± 0.6	(5)	2.3	± 0.7	(5)	2.2	± 0.4	(5)
PLT[23](K/μL)	1118	± 124	(5)	1176	± 98	(5)	1062	± 218	(5)	1129	± 155	(5)	1084	± 185	(5)	1064	± 76	(5)
MPV[24](fL)	6.2	± 0.9	(5)	6.3	± 0.7	(5)	6.2	± 0.7	(5)	6.2	± 0.6	(5)	6.6	± 1.0	(5)	6.2	± 1.0	(5)

Mean±S.D (Number of animals)

1: White blood cell, 2: Neutrophil, 3: Eosinophil, 4: Basophil, 5: Lymphocyte, 6: Monocyte, 7: Large unstained cell, 8: Percent of neutrophil, 9: Percent of eosinophil, 10: Percent of basophil, 11: Percent of lymphocyte, 12: Percent of monocyte, 13: Percent of large unstained cell, 14: Red blood cell, 15: Hemoglobin, 16: Red cell distribution width, 17: Hematocrit, 18: Mean corpuscular volume, 19: Mean corpuscular hemoglobin, 20: Mean corpuscular hemoglobin concentration, 21: Reticulocyte, 22: Methemoglobin, 23: Platelet, 24: Mean platelet volume

Table 8. Serum biochemical values of male rats in 28-day repeated dermal toxicity test.

WEEK	GROUP(㎎/㎏/day)																							
	G1(0)					G2(10)					G3(30)					G4(100)					G5(300)					G6(1,000)				
AST[1](IU/L)	129	±	31	(5)		113	±	19	(5)		108	±	25	(5)		111	±	17	(5)		119	±	39	(5)		107	±	19	(5)	
ALT[2](IU/L)	39	±	5	(5)		34	±	4	(5)		36	±	7	(5)		35	±	5	(5)		38	±	7	(5)		31	±	3	(5)	
GGT[3](IU/L)	0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)	
ALP[4](IU/L)	477	±	147	(5)		467	±	80	(5)		441	±	62	(5)		497	±	59	(5)		448	±	64	(5)		449	±	40	(5)	
BA[5](μmol/L)	27	±	16	(5)		22	±	6	(5)		13	±	3	(5)		26	±	13	(5)		18	±	10	(5)		24	±	19	(5)	
BIL[6](mg/dL)	0.01	±	0.01	(5)		0.01	±	0.01	(5)		0.00	±	0.01	(5)		0.02	±	0.01	(5)		0.01	±	0.01	(5)		0.02*	±	0.01	(5)	
BUN[7](mg/dL)	13.0	±	1.0	(5)		11.4	±	1.3	(5)		13.8	±	0.6	(5)		12.4	±	4.1	(5)		10.5	±	3.0	(5)		11.7	±	2.2	(5)	
CRE[8](mg/dL)	0.42	±	0.04	(5)		0.39	±	0.02	(5)		0.41	±	0.03	(5)		0.42	±	0.06	(5)		0.40	±	0.04	(5)		0.38	±	0.02	(5)	
UA[9](mg/dL)	1.2	±	0.5	(5)		0.9	±	0.3	(5)		1.2	±	0.4	(5)		1.0	±	0.5	(5)		1.1	±	0.3	(5)		1.1	±	0.5	(5)	
GLU[10](mg/dL)	165	±	29	(5)		164	±	38	(5)		185	±	25	(5)		161	±	20	(5)		166	±	26	(5)		176	±	32	(5)	
CHO[11](mg/dL)	69	±	19	(5)		78	±	14	(5)		76	±	19	(5)		67	±	15	(5)		57	±	15	(5)		73	±	22	(5)	
TG[12](mg/dL)	31	±	8	(5)		40	±	21	(5)		34	±	13	(5)		22	±	4	(5)		48	±	38	(5)		28	±	14	(5)	
PRO[13](g/dL)	6.2	±	0.4	(5)		6.3	±	0.4	(5)		6.2	±	0.2	(5)		6.3	±	0.2	(5)		6.1	±	0.3	(5)		6.2	±	0.3	(5)	
ALB[14](g/dL)	2.5	±	0.2	(5)		2.5	±	0.1	(5)		2.4	±	0.1	(5)		2.5	±	0.0	(5)		2.5	±	0.2	(5)		2.5	±	0.1	(5)	
A/Gratio[15]	0.66	±	0.02	(5)		0.66	±	0.05	(5)		0.64	±	0.03	(5)		0.67	±	0.03	(5)		0.70	±	0.07	(5)		0.68	±	0.06	(5)	
LDH[16](IU/L)	1533	±	710	(5)		1225	±	494	(5)		1215	±	646	(5)		1220	±	476	(5)		1315	±	951	(5)		1098	±	606	(5)	
CPK[17](U/L)	808	±	361	(5)		644	±	171	(5)		561	±	184	(5)		608	±	147	(5)		658	±	453	(5)		542	±	281	(5)	
ChE[18](U/L)	140	±	32	(5)		107	±	17	(5)		165	±	62	(5)		132	±	34	(5)		96	±	9	(5)		129	±	28	(5)	
Ca[19](mg/dL)	9.1	±	0.4	(5)		9.4	±	0.2	(5)		9.2	±	0.5	(5)		9.0	±	0.4	(5)		9.0	±	0.5	(5)		9.0	±	0.2	(5)	
IP[20](mg/dL)	7.3	±	0.3	(5)		7.9	±	0.7	(5)		7.5	±	0.6	(5)		7.6	±	0.5	(5)		8.0	±	0.2	(5)		7.7	±	0.1	(5)	
Mg[21](mg/dL)	2.1	±	0.1	(5)		2.0	±	0.2	(5)		2.3	±	0.4	(5)		2.1	±	0.2	(5)		1.9	±	0.2	(5)		2.0	±	0.3	(5)	
Na[22](mmol/L)	146	±	1	(5)		146	±	1	(5)		146	±	1	(5)		146	±	1	(5)		147	±	1	(5)		149**	±	1	(5)	
K[23](mmol/L)	5.1	±	0.1	(5)		5.0	±	0.3	(5)		4.8	±	0.1	(5)		4.8	±	0.2	(5)		4.9	±	0.2	(5)		4.9	±	0.2	(5)	
Cl[24](mmol/L)	106	±	1	(5)		105	±	1	(5)		105	±	1	(5)		105	±	1	(5)		106	±	2	(5)		107	±	1	(5)	

Mean±S.D (Number of animals)

1: Aspartate aminotransferase, 2: Alanine aminotransferase, 3: Gamma(γ)-glutamyl transferase, 4: Alkaline phosphatase, 5: Bile acid, 6: Total bilirubin, 7: Blood urea nitrogen, 8: Creatinine, 9: Uric acid, 10: Glucose, 11: Total cholesterol, 12: Triglyceride, 13: Total protein, 14: Albumin, 15: Albumin/Globulin ratio, 16: Lactate dehydrogenase, 17: Creatine phosphokinase, 18: Cholinesterase, 19: Calcium, 20: Inorganic phosphorus, 21: Magnesium, 22: Sodium, 23: Potassium, 24: Chloride

*: Significant difference compared with control group value, $p<0.05$ **: Significant difference compared with control group value, $p<0.01$

Table 9. Serum biochemical values of female rats in 28-day repeated dermal toxicity test.

WEEK	GROUP(㎎/㎏/day)																							
	G1(0)					G2(10)					G3(30)					G4(100)					G5(300)					G6(1,000)				
AST[1](IU/L)	128	±	23	(5)		117	±	20	(5)		122	±	13	(5)		115	±	10	(5)		125	±	45	(5)		117	±	37	(5)	
ALT[2](IU/L)	28	±	3	(5)		31	±	7	(5)		28	±	4	(5)		28	±	4	(5)		28	±	10	(5)		27	±	4	(5)	
GGT[3](IU/L)	0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)		0.0	±	0.0	(5)	
ALP[4](IU/L)	233	±	57	(5)		272	±	65	(5)		221	±	46	(5)		280	±	98	(5)		264	±	50	(5)		211	±	44	(5)	
BA[5](μmol/L)	12	±	5	(5)		22	±	15	(5)		22	±	10	(5)		21	±	18	(5)		16	±	15	(5)		19	±	8	(5)	
BIL[6](mg/dL)	0.02	±	0.01	(5)		0.02	±	0.01	(5)		0.04	±	0.01	(5)		0.04*	±	0.01	(5)		0.04	±	0.02	(5)		0.05*	±	0.02	(5)	
BUN[7](mg/dL)	13.5	±	2.3	(5)		13.1	±	1.1	(5)		12.8	±	0.9	(5)		11.8	±	4.5	(5)		10.1	±	2.9	(5)		12.7	±	2.9	(5)	
CRE[8](mg/dL)	0.38	±	0.01	(5)		0.40	±	0.02	(5)		0.40	±	0.01	(5)		0.40	±	0.05	(5)		0.38	±	0.03	(5)		0.38	±	0.04	(5)	
UA[9](mg/dL)	1.5	±	0.4	(5)		1.5	±	0.4	(5)		1.4	±	0.3	(5)		1.2	±	0.6	(5)		1.4	±	0.2	(5)		1.4	±	0.3	(5)	
GLU[10](mg/dL)	134	±	19	(5)		166	±	21	(5)		154	±	14	(5)		154	±	23	(5)		154	±	24	(5)		150	±	12	(5)	
CHO[11](mg/dL)	53	±	12	(5)		67	±	17	(5)		74*	±	18	(5)		63	±	13	(5)		43	±	11	(5)		59	±	11	(5)	
TG[12](mg/dL)	8	±	5	(5)		8	±	4	(5)		13	±	6	(5)		7	±	4	(5)		10	±	8	(5)		9	±	4	(5)	
PRO[13](g/dL)	6.1	±	0.5	(5)		6.2	±	0.2	(5)		6.4	±	0.3	(5)		6.3	±	0.2	(5)		6.3	±	0.3	(5)		6.3	±	0.3	(5)	
ALB[14](g/dL)	2.6	±	0.1	(5)		2.7	±	0.1	(5)		2.9*	±	0.1	(5)		2.6	±	0.2	(5)		2.6	±	0.1	(5)		2.6	±	0.2	(5)	
A/Gratio[15]	0.76	±	0.08	(5)		0.76	±	0.04	(5)		0.81	±	0.04	(5)		0.72	±	0.07	(5)		0.71	±	0.05	(5)		0.71	±	0.05	(5)	
LDH[16](IU/L)	1616	±	560	(5)		1525	±	498	(5)		1589	±	342	(5)		1299	±	496	(5)		1477	±	1015	(5)		1441	±	831	(5)	
CPK[17](U/L)	736	±	248	(5)		750	±	219	(5)		799	±	139	(5)		655	±	295	(5)		680	±	400	(5)		746	±	488	(5)	
ChE[18](U/L)	811	±	198	(5)		1013	±	273	(5)		1082	±	206	(5)		981	±	203	(5)		783	±	377	(5)		909	±	578	(5)	
Ca[19](mg/dL)	8.8	±	0.2	(5)		8.9	±	0.1	(5)		8.9	±	0.1	(5)		8.5	±	0.5	(5)		8.8	±	0.4	(5)		8.7	±	0.4	(5)	
IP[20](mg/dL)	6.7	±	0.5	(5)		6.8	±	0.9	(5)		6.8	±	0.6	(5)		6.4	±	0.7	(5)		6.6	±	0.4	(5)		6.4	±	0.5	(5)	
Mg[21](mg/dL)	1.8	±	0.1	(5)		1.8	±	0.2	(5)		2.0	±	0.4	(5)		2.1	±	0.2	(5)		1.8	±	0.1	(5)		1.8	±	0.2	(5)	
Na[22](mmol/L)	144	±	1	(5)		144	±	1	(5)		145	±	1	(5)		146	±	2	(5)		147**	±	1	(5)		147**	±	1	(5)	
K[23](mmol/L)	4.7	±	0.3	(5)		4.8	±	0.1	(5)		4.8	±	0.3	(5)		4.6	±	0.3	(5)		4.8	±	0.2	(5)		4.8	±	0.5	(5)	
Cl[24](mmol/L)	106	±	2	(5)		105	±	1	(5)		106	±	1	(5)		106	±	2	(5)		108	±	1	(5)		108	±	1	(5)	

Mean±S.D (Number of animals)

1: Aspartate aminotransferase, 2: Alanine aminotransferase, 3: Gamma(γ)-glutamyl transferase, 4: Alkaline phosphatase, 5: Bile acid, 6: Total bilirubin, 7: Blood urea nitrogen, 8: Creatinine, 9: Uric acid, 10: Glucose, 11: Total cholesterol, 12: Triglyceride, 13: Total protein, 14: Albumin, 15: Albumin/Globulin ratio, 16: Lactate dehydrogenase, 17: Creatine phosphokinase, 18: Cholinesterase, 19: Calcium, 20: Inorganic phosphorus, 21: Magnesium, 22: Sodium, 23: Potassium, 24: Chloride

*: Significant difference compared with control group value, $p<0.05$ **: Significant difference compared with control group value, $p<0.01$

Absolute organ weight measurements displayed decreased right ovary weights in the female 10 and 100 mg/kg/day treatment groups, and decreased brain weight in all 100 mg/kg/day treatment groups. Relative organ weight measurements exhibited decreased right ovary weights in the 10, 100, and 1,000 mg/kg/day treatment groups, and elevated thymus weights in the female 300 mg/kg/day treatment group. The reduction in absolute and relative weights of the right ovary in all female treatment groups was not attributed to BPF as there was no dose–response correlation and no related histopathological findings were detected. Other changes, such as decreased absolute brain weight and increased relative weight of the thymus, did not display dose–response correlations, and thus not attributed to BPF exposure

Histopathological testing showed multifocal inflammatory bronchioalveolar cell infiltrations in the lungs, and ultimobranchial cysts in the thyroid glands of female rats. In male rats, multifocal mononuclear cell infiltrations were found in the liver. Further, localized focal basophilic tubules and localized focal mononuclear cell infiltrations were noted in the renal cortex and heart, respectively. Histopathological testing demonstrated mild mononuclear cell infiltration (liver and heart), basophilic tubules (kidney, thyroid sac, periportal vacuolization, kidney sac, and pituitary coronary remnants), and inflammatory cell infiltration (lung bronchioles), as well as background lesions (Boorman et al. 1990) that did not differ significantly in frequency among the groups. Thus, evidence indicates that BPF-related changes were not detected (Figure 7).

Thus, our findings suggest that there were no marked toxicological changes following transdermal administration of BPF to rats for 4 weeks, followed by a recovery period of 14 days. Therefore, the NOAEL of BPF in this study was considered to be 1,000 mg/kg/day.

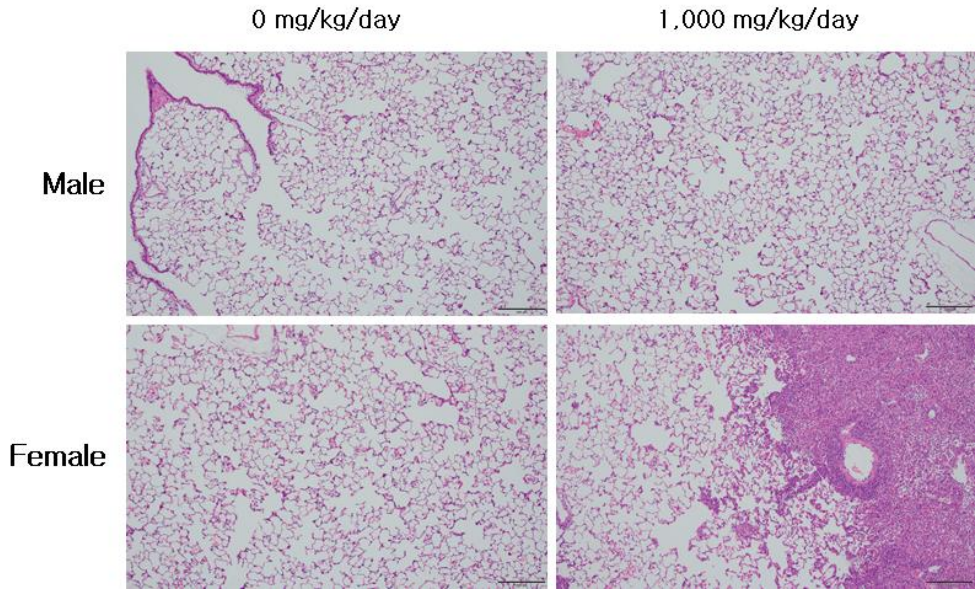

Figure 7. Findings seen in histopathology examination.

H&E-stained slide of inflammatory cell infiltration into lung tissue in female rats. This histopathological finding was not considered Bisphenol F administration, because the increased frequency of lesions was only found a one-off case in the female group and the severity was low. Additionally, bronchioloalveolar cell infiltration was commonly observed in SD rats, especially in the long-term toxicity study. Original magnification, 200′, scale bar: 200 μm.

5. Skin and eye irritation tests

Evaluation of abraded and non-abraded skin reactions 24 and 72 hr after BPF administration did not exhibit erythema, crust formation, or edema. Further, evaluation of skin irritation 24 and 72 hr after BPF administration displayed a Primary Irritation Index of 0. Therefore, in this study, the final skin irritation grade of BPF was classified as a non-irritant (Table 10, Figure 8).

Table 10. Evaluation for skin irritation.

Group	Erythema and Eschar formation				Edema				PII*
	Intact Skin		Abraded Skin		Intact Skin		Abraded Skin		
BPF treated group	Total	0	Total	0	Total	0	Total	0	0.0
	Mean	0.0	Mean	0.0	Mean	0.0	Mean	0.0	
Negative control	Total	0	Total	0	Total	0	Total	0	0.0
	Mean	0.0	Mean	0.0	Mean	0.0	Mean	0.0	

(1) Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4
(2) Edema formation	
No edema	0
Very slight edema(barely perceptible)	1
Well-defined edema(edges of area well-defined by definite raising)	2
Moderate edema(raised approximately 1 millimeter)	3
Severe edema(raised more than 1 millimeter and extending beyond exposure area)	4

* PII(Primary Irritation Index) = Total score of skin response/4

In the wash group of the eye irritation test, conjunctival redness, edema, and discharge were observed until day 7 after administration. In the non-wash group, corneal opacity, iris reaction, conjunctival redness, edema, and discharge were found until day 7. The index of acute ocular irritation was 12 and 33.5 for wash and non-wash groups, respectively. All ocular mucosal irritation was resolved within 18 days of administration. Therefore, in this study, the final ocular irritation grade of BPF was classified as a mild to moderate eye irritant with a reversible response (Table 11, Figure 8).

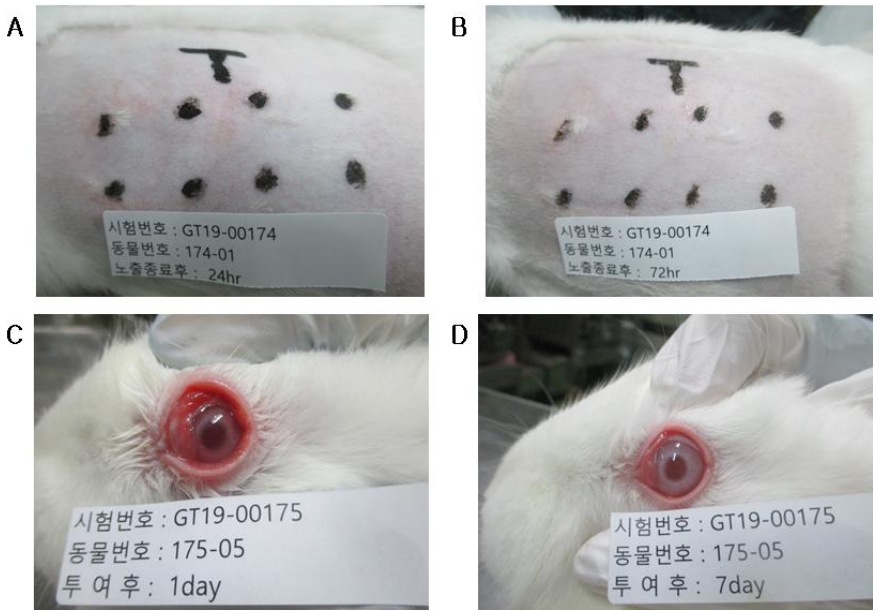

Figure 8. Skin/Eye irritation reaction in rabbits after application of Bisphenol F.

A) Observation of skin irritation in BPF administration group at 24 hours. As a result of observing abrasion and non-abrasion skin 24 hours after administration of the test substance, erythema and crusting were not observed. B) Observation of skin irritation in BPF administration group at 72 hours. As a result of observing abrasion and non-abrasion skin 48 hours after administration of the test substance, erythema and crusting were not observed. C) Observation of eye irritation in BPF administration group at 1 day. As a result of observation of the eye, corneal opacity, conjunctival redness, edema, and discharge were observed on the 1 day after administration. D) Observation of eye irritation in BPF administration group at 7 day. As a result of observation of the eye, corneal opacity, conjunctival redness, edema, and discharge were observed on the 7 day after administration. All eye irritation caused by BPF was recovered on the 18th day of administration.

Table 11. Evaluation for eye irritation.

Group		MIOI *						IAOI **
		1day	2day	3day	4day	7day	18day	
Rinsed group	BPF group	12.0	9.3	8.7	6.7	4.0	0.0	12.0
		3/3***	3/3	3/3	3/3	2/3	0/3	
	control group	0.0	0.0	0.0	0.0	0.0	0.0	
		0/3	0/3	0/3	0/3	0/3	0/3	
Non-rinsed group	BPF group	25.7	33.5	29.8	19.3	17.2	0.0	33.5
		6/6	6/6	6/6	6/6	6/6	0/6	
	control group	0.0	0.0	0.0	0.0	0.0	0.0	
		0/6	0/6	0/6	0/6	0/6	0/6	

(1) Cornea
(A) Opacity : Degree of Density(area which is most dense is taken for reading)
Scattered or diffuse area-details of iris clearly visible 1
Easily discernible translucent areas. Details of iris slightly obscured 2
Opalescent areas no details of iris visible 3
Opaque. Iris invisible 4
(B) Area of Cornea Involved
One quarter (or less) but not zero 1
Greater than one quarter-less than one-half 2
Greater than one one-half less than three quarters 3
Greater than one quarters up to whole area 4
(2) Iris
(A) Values
Folds above normal, congestion, swelling, circumcorneal injection 1
No reaction to light, hemorrhage, gross destruction (any one or all of these) 2
(3) Conjunctiva
(A) Redness(refers to palpebral conjunctivae only)
Vessels definitely injected above normal 1
Vessels definitely congestion 2
More diffuse deeper crimson red individual vessels not easily discernible 3
Diffuse beefy red 4
(B) Edema
Any swelling above normal (includes nictitating membrane) 1
Obvious swelling with partial eversion of lids 2
Swelling with lids about half closed 3
Swelling with lids about half closed to completely closed 4
(C) Discharge
Any amount different from normal 1
Discharge with moistening of the lids and hairs just adjacent to the lids 2
Discharge with moistening of the lids and considerable area around the eye 3

* MIOI (Mean index of ocular irritation): from Day 1 to Day 7.
** IAOI (Index of acute ocular irritation) = maximum value of MIOI.
*** Number of animals with skin reactions/Total animals.

6. Skin sensitization test

During the experiment, no adverse general symptoms, dead animals, or abnormalities in body weight change were observed following BPF administration. However, general symptoms and skin reactions were detected 24 and 48 hr after removing the induction patch. Further, 7 out of 10 BPF-treated rats displayed a level 1 or higher reaction (erythema scattered at the application site) (Table 12 and Figure 9). Therefore, the final sensitization rate of BPF was 70%, which classifies it as a strong (grade IV) inducer of skin sensitization, according to Magnusson and Kligman (1969) skin sensitization according to OECD criteria (1992).

Table 12. Summary of skin sensitization test results.

Group	No. of animals showing score				Sensitization rate	
	0*	1	2	3	Fraction	%
BPF treated group	3	2	5	0	7/10	**70**
Negative control	5	0	0	0	0/5	0
Positive control	1	1	3	0	4/5	**80**

* Score interpretation: 0, no visible change; 1, discrete or patchy erythema; 2, moderate and confluent erythema; 3, intense erythema and swelling.

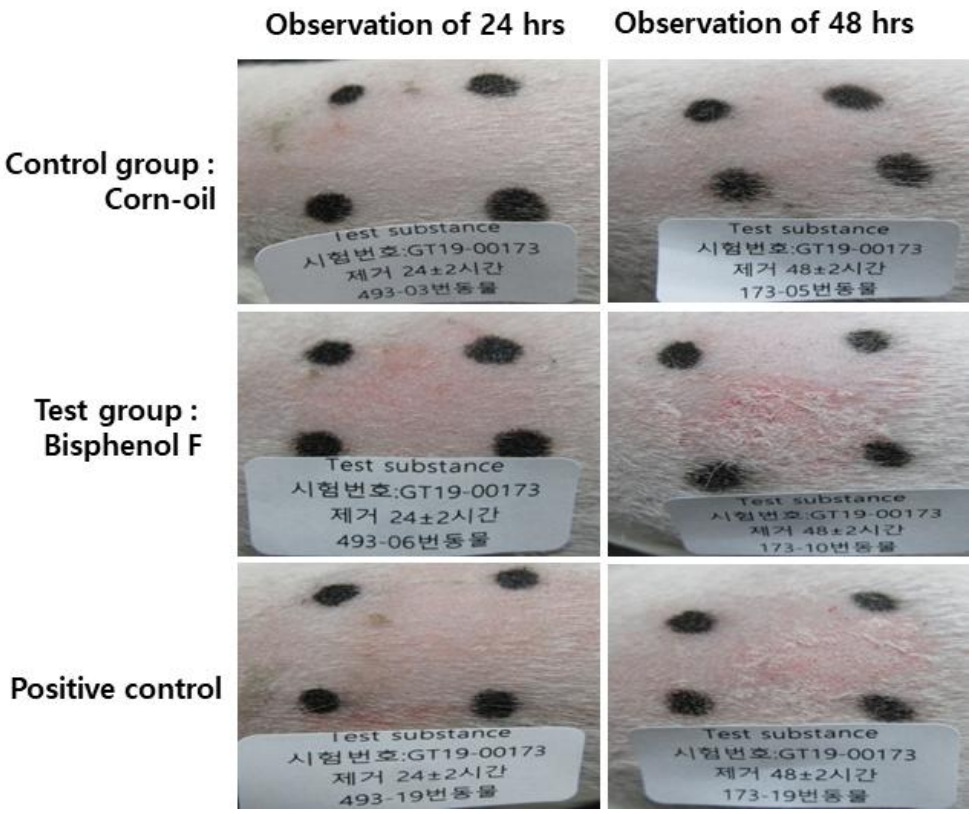

Figure 9. Delayed hypersensitivity reaction in skin sensitization test.

In the case of control group, sensitization rates were 0 % at both 24, 48 hours and the control group was classified as "Weak" (Grade I) sensitiser. In the case of BPF treated group, sensitization rates were 70 % at 24, 48 hours and the BPF was classified as "Strong" (Grade IV) sensitiser. In the case of Positive control group, sensitization rates were 80 % at 24, 48 hours and the positive control group was classified as "Strong" (Grade IV) sensitiser.

Discussion

Recently, not only BPA but also BPF is present everywhere in the environment where people live due to the mass production and widespread application. The most predominant route for human exposure to BPF is the gastrointestinal and inhalation tract. However, for workers who directly deal with bisphenol, such as possibility through thermal paper or occupational exposure, access through the skin should be treated as very important. In particular, thermal paper is regarded as an important source of BPA that cannot be ignored in certain occupational groups (cashiers), and BPA intake was estimated to be 0.0511 μg/kg/day in the college-age population by handling thermal paper (Bernier and Vandenberg, 2017). This is similar to the total human exposure of 0.01～0.05 μg/kg/day in the integrated risk assessment for bisphenols (BPA, BPS, BPF) conducted by the Ministry of Food and Drug Safety in 2020. Therefore, Skin exposure to BPF is a prominent routes of absorption in humans. Further, the amount of BPF that remains on the skin, and extent and pattern of absorption or penetration into the skin, have not yet apparently been reported. In this study, the degree with which BPF was absorbed by, and able to penetrate into, the skin was quantitatively analyzed. Considering the BPF skin absorption rate data, the possibility of BPF skin contact inducing toxicity was determined. Extensive skin-related toxicity testing was conducted and the results analyzed.

Both hydrophilic and lipophilic properties are required for skin penetration. Highly hydrophilic molecules cannot undergo transdermal delivery, whereas excessive lipophilic molecules tend to remain in the transdermal layer (Kim et al. 2018). Flux (μg/h/cm²) value and Kp (cm/hr) value were used to interpret the skin absorption results, and each value means the inherent permeation characteristics of the material. The permeation coefficient Kp (cm/hr) is calculated as the value obtained by dividing the initial concentration value of the substance from the Flux (μg/h/cm²) value, which is the calculated amount of the sample that penetrates the skin in a

constant area per unit time. This values are calculated to analyze the permeation characteristics of the material.

The mean residual amount in the skin, and mean absorbed dose of BPF were 1.7% (absorption amount 0.11 mg), and 26.5% (absorption amount 1.64 mg) and Benzoic acid were 1.7% (absorption amount 0.11 mg), and 80.9% (absorption amount 1.64 mg). Therefore, the skin absorption test using BPF and benzoic acid demonstrated 26.5 and 80.9% skin absorption rates, respectively, with cumulative permeation increasing with time. The skin permeability of a substance may be characterized by flux and Kp values. BPF showed numerically higher flux and Kp values than benzoic acid. According to the calculation method by Marzulli et al (1969), the permeation coefficient (Kp, cm/hr) was categorized as “very fast”(Kp : 1.3E-01) and “fast” (Kp : 2.2E-02) for benzoic acid and BPF, respectively.

Compared to the results of previous studies investigating the skin absorption of other bisphenol analogs (Champmartin et al., 2020; Toner et al., 2018), The mean residual amount in the skin, and mean absorbed dose of BPA were 27% (BPF 1.7 %), and 41% (BPF 26.5%) and BPS were 47% (BPF 1.7 %), and 1% (BPF 26.5%). The residual amount in the skin of BPF was lower than that of BPA and BPS and absorbed dose of BPF was higher than BPS, but lower than BPA (Table 13). Therefore, the amounts of BPF absorbed in the epidermis and dermis lower than those of BPA and BPS. As a resulf of analysis of the permeability of bisphenol analogue(BPF, BPA, BPS) according to Marzulli fomula (Marzulli, 1969), BPF had significantly lower flux and Kp values, both of which are indicators of permeation speed, than BPA and BPS (Figure 10).

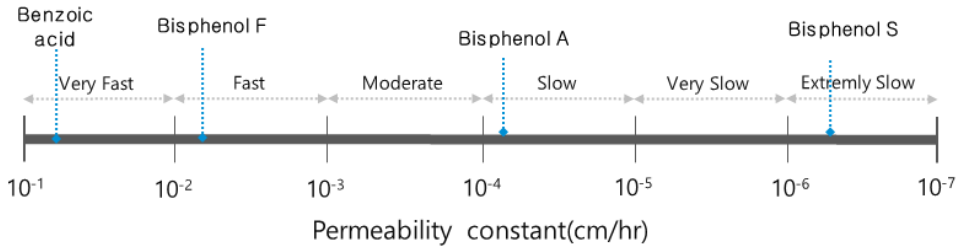

Figure 10. Comparison of penetration degree in bisphenol analogues (BPF, BPA, BPS).

Analysis of the permeability of bisphenol analogue(BPF, BPA, BPS) according to Marzulli fomula (Marzulli, 1969). As a result, the BPF permeability constant(Kp) was 2.2 x 10^{-2} and the BPA Kp was 9.3 x 10^{-5}, the BPS Kp was 2.6 x 10^{-6}. Therefore, BPF showed fast, BPA showed slow and BPS showed extremly slow.

Table 13. Comparing our results to those of previous studies investigating the skin absorption of other bisphenol analogues.

Absorption profile		% Applied dose			
		BPA	BPS	BPF	Benzoic acid
Absorption rate	Skin	27 %	47 %	1.7 %	1.7 %
	Absorbed dose	41 %	1 %	26.5 %	80.9 %
	Dermal delivery*	68 %	48 %	28.2 %	82.6
Flux (μg/h/㎠)		0.0372 ± 0.0057	0.001 ± 0.0002	136.7 ± 101.2	805.0 ± 405.2
Kp (cm/h)		9.30E-05	2.60E-06	2.2E-02	1.3E-01

*Dermal delivery = Skin + Absorbed dose

The NOAEL(no observed adverse effect level) for dermal application of BPF was determined to be 1,000 mg/kg/day as adverse effects on target organs were not observed. In toxicology, a dose of 1,000 mg/kg/day is the limiting dose, and concentrations above the limiting dose have no toxicological significance. That is, BPF is a completely non-toxic substance that does not show toxicity even when administered at the maximum toxicologically significant dose.

However, contrary to the results of this study, Higashihara et al (2007) examined different routes of administration by orally giving BPF for 28 days at doses of control 0, 20, 100 or 500 mg/kg/day and reported significant toxic changes in hematochemical parameters. In addition, liver toxicity was observed based upon liver weight with no associated histopathological changes. In female rats, weight loss and a decrease in total cholesterol, glucose, and albumin were recorded. The oral NOAEL was determined to be 20 mg/kg/day, and the target organ was found to be the liver. These findings were postulated to result from the varied BPF absorption rates associated with oral administration. When the absorption of bisphenol analogues are exposed through the skin, it has been found that the absorption amount is about 10% of the amount absorbed through oral exposure (ECB 2008). In addition, considering the low skin absorption of BPF among the bisphenol analogues found as a result of this study, it is considered that the amount absorbed into the body through transdermal administration is a very low amount that cannot reach the level causing toxicity. According to the integrated risk assessment report for bisphenol analogues (BPA, BPS, BPF) conducted by the Ministry of Food and Drug Safety in Korea in 2020, the total exposure in the human body was found to be 0.01～0.05 μg/kg/day in all age groups. This is a very low concentration compared to the NOAEL concentration of 20 mg/kg/day, and it is thought that the concentration exposed in real life cannot cause toxicity through transdermal administration. To further confirm this finding, future pharmacokinetic studies investigating BPF and various routes of administration are necessary.

As BPF has a similar structure to that of BPA, the potential to disrupt the endocrine system has been highlighted in previous studies demonstrating thyroid endocrine disruption, estrogenic and anti-androgenic activity early development and growth similar activity to BPA (Basak, Das, and Duttaroy 2020; Den Braver-sewradj, Van Spronsen, and Hessel 2020; Ullah et al. 2018; Wu et al. 2018; Zhang et al. 2017). However, in the

28-day toxicity test, in which BPF was repeatedly administered orally (Higashihara et al. 2007), no specific abnormalities were observed in the estrus cycle test evaluating estrogenic activity. Further, serum thyroid-stimulating hormone, thyroxin, and triiodothyronine exhibited no abnormal activity. Our findings are in agreement that oral BPF in vivo did not produce any marked adverse alterations in several metabolic and histopathological parameters.

The skin sensitization test evaluates whether contact dermatitis is initiated by a delayed hypersensitivity reaction (Type 4 hypersensitivity reaction). In this study, administration of about 6 mg/kg concentration BPF produced a strong (Level IV) sensitization reaction with a sensitization rate of 70%. The concentration(6 mg/kg) used in this study is very high compared to the concentration(0.01～0.05 μg/kg/day) of total exposure in the human body. But, Workers using bisphenol are expected to have higher than normal exposures. Although no apparent previous studies examined skin sensitization effects of BPF except for a study by Bruze (1986) but Using a 20-year follow-up human baseline patch test, some investigators reported a contact allergy in 66 (3.3%) out of 1972 workers handling BPF epoxy resins (Aalto-Korte et al. 2014; O'Boyle et al. 2012). Although the present study indicated that sensitization via direct skin exposure to BPF warrants consideration, further research is needed to identify the mechanisms through which BPF elicits such a response.

This experiment determined that it causes allergic contact dermatitis (skin sensitization test), and follow-up studies on workers will be needed to clarify this. A preliminary study (Yamazaki, 2015) identified that bisphenol F epoxy resin was widely used as a coating agent for water pipes in Japan, South Korea, and China, and BPF detected in rivers in Japan, Korea, and China was more than twice as high as in other countries. Therefore, it will be necessary to conduct further long-term monitoring study on screening occupational contact allergy to bisphenol F epoxy resin for workers who participated in producing bisphenol F epoxy resin in Japan, Korea, and China.

The eye mucosa irritation test showed that BPF produced mild to moderate irritation. However, as oral consumption and the skin present the main routes through which BPF exposure occurs, irritation via the eyes is considered a lower risk. This study demonstrates that BPF is not a skin irritant.

Conclusions

This study assessed the degree and rate at which BPF is absorbed onto the skin followed by pattern of absorption, The degree of skin absorption rate was determined for BPF and benzoic acid of each found to be 26.5% and 80.9%, respectively. Further, BPF was found to penetrate into the subcutaneous layer at a “fast rate”(Kp : 2.2E-02) similar to that of benzoic aicd (very fast rate, Kp : 1.3E-01). As a result of single dermal administration was performed on SD rats, no toxic symptoms were observed, and the approximate lethal dose was judged to exceed 1,000 mg/kg in both males and females. In case of repeated dermal toxicity tests, no toxicological change due to the test substance was observed. The non-toxic content of this test substance was judged to be 1,000 mg/kg/day, and no target organ was observed. Additionally, the final skin/eye irritation grade of BPF was classified as a non-irritant. Therefore, Extensive toxicological evaluations of BPF exposure to skin showed no adverse toxicological changes.

In contrast, skin sensitization testing indicated that BPF induced strong sensitization similar to that of hydroxycitric acid (HCA). In this study, no BPF-initiated toxicity symptoms were observed in various toxicity assays. However, BPF was found to induce a strong hypersensitivity reaction in the skin following contact likely due to its high rate of skin permeation. The findings in this study may be used as baseline data for future research assessing the effects of BPF on the immune system.

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Summary in Korea

생활화학물질 중 비스페놀 F는 비스페놀 A의 대체제로써 식품용기, 상수도관, 영수증 감열지로 사용되고 있으며, 실제 우리나라에서도 식수 및 인간 요 시료에서 검출되고 있다. 그러나 비스페놀 F에 대한 독성정보에 대한 연구가 제한적이기 때문에 적절한 사용지침을 마련하기 어려우며 사회적 불안감이 증폭되고 있다.
최근 연구에서 비스페놀F 역시 내분비계 교란물질로 지목되고 있으며, 실제로 관련 위해성이 연구로 밝혀지고 있다. 그러나 이러한 연구들은 직접 섭취 또는 흡입에 의한 BPF 인체 노출에 초점을 맞춘 연구들이 대부분이며 피부 접촉을 중심으로 BPF의 독성학적 영향을 평가한 연구는 부족한 실정이다. 따라서 이 연구에서 우리는 피부에 투여될 수 있는 독성시험들에 초점을 맞추어 광범위한 독성 시험을 진행하였다. 생체외 적용을 통하여 피부에 흡수되는 정도와 투과되는 패턴을 평가하였다. 피부 노출시의 무독성량(NOAEL) 및 표적장기를 규명하기 위하여 단회투여 경피독성시험, 14일반복용 경피투여 용량결정시험, 28일 반복 경피독성시험을 수행하였다. 피부 노출시 국소적인 자극성을 알아보기 위하여 피부자극시험, 눈자극시험을 수행하였으며, 피부 접촉에 따른 지연성 과민반응의 평가를 위한 피부감작시험을 실시 하였다.

결과는 BPF가 다른 비스페놀 유사체에 비해 표피 또는 진피에 남아있는 BPF의 양이 더 높은 비율로 피부를 통해 흡수되는 것으로 나타으며 BPF는 매우 빠른 속도로 피하층에 침투하는 것을 확인 하였다. BPF에 피부 노출 후 독성학적 변화나 국소 자극은 관찰되지 않았다. 그러나 BPF는 양성(HCA)과 유사한 강한 감작을 유도하는 것을 확인 하였다..

본 연구의 결과는 BPF의 높은 피부 침투율 및 피부 흡수 결과를 통해 피부 노출의 유의미한 함의를 보여주었고, BPF에 대한 피부 노출에 대한 광범위한 독성학적 평가 결과 BPF가 피부 감작을 유발함을 확인하였다.