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Benzotriazole Ultraviolet Absorber Contamination in Breast Milk and the Infant Health Risk

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The contamination levels of benzotriazole ultraviolet absorbers (BUVAs) were determined in breast milk samples from 36 Japanese mothers. BUVAs were detected in all samples in the ranges of 11.0-803 ng/g lipid weight. Especially, 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl) phenol (UV-320) accounted for 36% of the total. The BUVAs levels were comparable to some previous reports. The total BUVAs levels in this study were higher than those of Vietnam and Korea. A comparison of primiparas and multiparas showed that the mean total BUVAs level in multiparas was 2.1 times higher than that in primiparas. In particular, UV-320 levels were significantly higher in multiparas than in primiparas ($p<0.05$). Subsequently, the estimated daily intake of infants was calculated to be 76.5-4,410 ng/kg/day. Further studies are needed to help develop regulations for these chemicals in our living environment and prevent harmful exposure.

Key words benzotriazole ultraviolet absorbers, breast milk, infant health risk, contamination

INTRODUCTION

Ultraviolet absorbers (UVAs) are materials that absorb ultraviolet light and release energy in the form of heat. They are commonly used to protect other materials from degradation by harmful UV rays, particularly in the highly damaging UV-A and UV-B regions. UVAs are broadly classified into benzotriazole, benzophenone, and triazine systems. The structural formula of benzotriazole-based UVAs (BUVAs) is shown in Fig. 1. BUVAs are used in a variety of products because they provide higher UV protection compared with benzophenone-based UVAs. Benzophenone-based UVAs are generally employed in woodworking applications, but their use is discouraged because they have been designated as environmental hormones.

In 2007, 2-(2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl) phenol (UV-320) was categorized as a class I chemical substance in Japan under the Chemical Substances Control

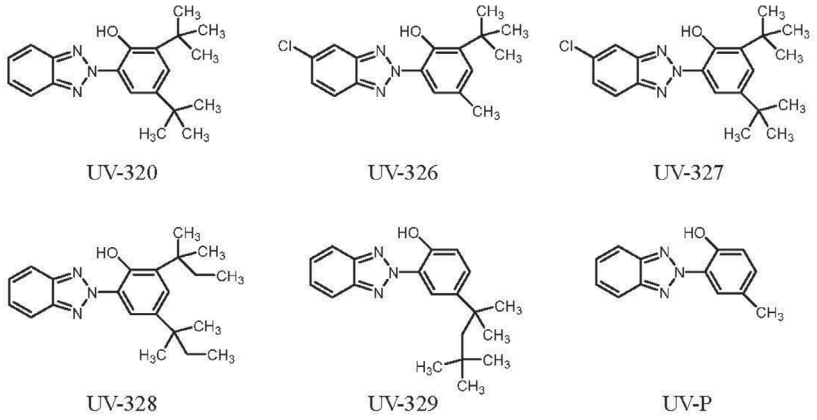

Fig. 1. Chemical Structures of the Benzotriazole Ultraviolet Absorbersmeasured in This Study

UV-320 is 2-(2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl) phenol. UV-326 is 2-(5-chloro-2*H*-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methylphenol. UV-327 is 2-(5-chloro-2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl) phenol. UV-328 is 2-(2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl) phenol . UV-329 is 2-(2*H*-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl) phenol. UV-P is 2-(2*H*-benzotriazol-2-yl)-4-methylphenol.

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Law by the Ministry of Economy, Trade and Industry.[1] The production, import, and use of class 1 substances are restricted.

In addition, 2-(5-chloro-2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl) phenol (UV-327) and 2-(2*H*-benzotriazol-2-yl)-4-(1,1-dimethylethyl)-6-(1-methylpropyl) phenol (UV-350) were designated as monitoring chemicals in 2004 and 2006, respectively. The manufacture, import, and use of these compounds must be reported annually. Furthermore, 2-(2*H*-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl) phenol (UV-328) was adopted for inclusion in Annex A at COP11 of the Stockholm Convention held in May 2023. It was decided that internationally coordinated efforts are necessary to eliminate its production and use in the future.

Moreover, four BUVAs, namely UV-320, UV-327, UV-328, and UV-350, are on the candidate list for Substances of Very High Concern under the EU REACH regulation, and products are regulated when they contain ≥ 0.1% of these substances. After November 27, 2023, these four BUVAs cannot be used without authorization, under Annex XIV of the REACH regulation. However, other BUVAs are currently unregulated, and there are no restrictions on their use or production.

Regarding toxicity reports on BUVAs, *in vivo* studies revealed that UV320 induces hepatotoxicity through PPARα signaling.[2] In vitro studies showed that 2-(5-chloro-2*H*-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methylphenol (UV-326) has AhR activation property and may exhibit toxicity similar to dioxin,[3] and UV-328 exhibits potent antiandrogenic activity after CYP3A4-mediated metabolism.[4] However, there are few reports on the toxicity of unregulated BUVAs, even though they likely have comparable toxicity, bioaccumulation, and environmental persistence because they have a similar chemical structure to UV-320 and show equivalent mass solubility.[5] Currently, there is limited information on the toxicity, human exposure, and adverse effects of BUVAs.

Breast milk is an important matrix for monitoring human exposure to environmental chemicals. It is also the main dietary source of xenobiotic exposure in infants. This study aimed to investigate the actual contamination status of BUVAs in breast milk of the Japanese population. Moreover, we also compared the BUVA levels in breast milk from several countries and between primiparas and multiparas. Finally, the estimated daily intake of BUVAs by the infants was calculated using the contamination level.

MATERIALS AND METHODS

Milk Samples Between June 2018 and May 2021, 36 breast milk samples were collected from healthy donors residing in Ibaraki, Osaka, Saitama, and Tochigi. The samples were provided three months after the birth of the child. Approximately 50 mL of whole milk were stored at -30°C for future analysis. The donors ranged from 26 to 42 years old. Additionally, the dietary preferences of each donor were assessed by conducting a questionnaire, which gauged their weekly intake of meat, seafood, vegetables, fruits, and dairy products. The data of the mothers included in this study are shown in Table 1. Prior to sampling, all mothers agreed on the purpose of the research according to ethical rules established by the Setsunan University Ethical Review Committee for Medical and Health Research Involving Human Subjects.

Table 1. Summary of Information Obtained from Questionnaires Handed Out to the Mothers

Participating mothers	36
Sampling period	June 2018 – May 2021
Mother's age	33.4 ± 3.9
Mother's body weight	55.3 ± 7.2
Mother's height	158.8 ± 4.8
Mother's BMI*	21.9 ± 2.4
Pregnancies	
Primiparous	14%
Multiparous	86%

*BMI: body mass index

Chemicals In this study, six BUVAs were measured. UV-320 was purchased from Fluorochem (Hadfield, UK). UV-326, UV-327, UV-328, 2-(2*H*-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl) phenol (UV-329), and 2-(2*H*-benzotriazol-2-yl)-4-methylphenol (UV-P) were purchased from Tokyo Chemical Industry (Tokyo, Japan). $^{2}H_{3}$-UV-326, $^{2}H_{20}$-UV-327, and $^{2}H_{12}$-UV-328 standards were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis HLB cartridges (500 mg, 6 cc), used for purification, were purchased from Waters (Milford, MA, USA). All other reagents and solvents were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Analysis Breast milk samples (5.0 mL) were spiked with 5.0 ng of $^{2}H_{3}$-UV-326, $^{2}H_{20}$-UV-327, or $^{2}H_{12}$-UV-328 in a glass flask. Subsequently, 10 mL of saturated sodium oxalate, 10 mL of ethanol, 15 mL of diethyl ether, and 15 mL of *n*-hexane were added and shaken using a shaker for 15 min. After separating the organic phase, diethyl ether and *n*-hexane were added to the lower phase, and the mixture was shaken again in the same manner. After mixing the two organic phases, they were washed with water, dehydrated, and filtered. This organic phase was dried with N_2 and weighed, and the lipid content was measured. After adding 2 mL of 1 mol/L potassium hydroxide/ethanol to the lipid, the mixture was sonicated. Another 13 mL of 1 mol/L potassium hydroxide/ethanol was added to make a total of 15 mL, and the lipids were shaken for 2 h for alkaline degradation. Subsequently, 30 mL of water and 25 mL of *n*-hexane were added and shaken for 15 min. The lower phase was removed, and 25 mL of *n*-hexane was added to this phase and shaken in the same manner, and this process was repeated twice. The organic phase was washed with water, dehydrated, and filtered. The extract was concentrated, and solid-phase extraction was performed using a Waters HLB cartridge. Purification was performed using a florisil column (2.0 g) and eluted with 15 mL of 5% ethyl acetate in *n*-hexane. The purified extract was then concentrated to 100 µL in *n*-nonane. The final solution was analyzed for BUVAs by GC/MS. The GC/MS setup consisted of an Agilent 6890N gas chromatograph (GC, Agilent Technologies, USA) coupled with a JEOL JMS-Q1500 mass spectrometer (MS, JEOL, Japan). The ion source was operated in the electron ionization mode (70 eV, 200 µA, 250°C). The sample extract was analyzed using a Trajan BP5MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The GC oven temperature was programmed as follows: the initial temperature of 100°C was maintained for 1.5 min, which was then increased to 200°C at a rate of 20°C min^{-1}, and finally, the temperature was increased at a rate of 15°C min^{-1} to achieve the final temperature of 320°C, which was

maintained for 4 min. The column was connected directly to the ion source of the mass spectrometer (interface temperature of 250°C). Sample introduction was performed by splitless injection (injection temperature of 250°C, splitless time of 1 min) of the sample extract (1.0 μL). Helium was used as the carrier gas at 1.0 mL/min.

Method of QA/QC for Analytical Data BUVAs were identified in the samples by comparing their retention times and mass spectra with those of the commercial standards. Furthermore, selected ion monitoring chromatograms were used to identify the peak area ratios for $[M\text{-}CH_3]^+$ / $[M]^+$. The acceptance criteria were set from −30% to 30% of the ratios observed using the commercial standards. Levels were corrected based on the recovery efficiency of three internal standards: $^2H_3$-UV-326, $^2H_{20}$-UV-327, and $^2H_{12}$-UV-328. Samples that had high internal standard recoveries in the range of 60%–120% were used for data collection. The limits of detection (LODs) and limits of quantification (LOQs) were defined as three (S/N = 3) and ten times (S/N = 10) the noise level, respectively. The LOQs of UV-320, -326, -327, -328, -329, and -P in breast milk were 0.040, 0.050, 0.060, 0.010, 0.110, and 0.090 ng/g lipid wt., respectively. When we estimated the daily intake of BUVAs through breast milk, values between LOD and LOQ were assumed to be 1/2 LOQ.

Statistical Analysis Statistically significant differences in mean values were observed using the Student's t-test. Analysis was carried out using JMP software. The p-values below 0.05 were considered to indicate significance.

RESULTS AND DISCUSSION

BUVA Levels in Breast Milk BUVAs were measured in the breast milk of healthy women residing in Japan, and the levels of each compound are shown in Table 2. ΣBUVAs represents the sum of UV-320, -326, -327, -328, -329, and -P. The average level of BUVAs in breast milk was 216 ng/g lipid wt. (range of 11.0–803 ng/g lipid wt., median of 119 ng/g lipid wt.). UV-320, -326, and -329 were detected in all samples. Considering the composition of each BUVA in breast milk, UV-320 accounted for 36% of the total, followed by UV-327 (20%) and -326 (15%). UV-328 had a relatively low level of 9.14 ng/g lipid wt. (4.2%).

Comparison of BUVA Levels in Breast Milk from Several Countries The BUVA levels found in breast milk in this study were comparable to those reported in previous studies,[6-9] as shown in Fig. 2. The total BUVAs represents the sum of six chemicals of UV-320, -326, -327, -328, -329, and -P for Vietnam, Korea, and this study. Moreover, four chemicals were reported in China (UV-326, -327, -328, and -P), four chemicals were reported in the Philippines (UV-320, -326, -328, and -P), and two chemicals were reported in Spain (UV-320 and -329). The total BUVA levels in this study were 4.8 times higher than those in Vietnam and 2 times higher than those in Korea. However, the total BUVAs are 586 ng/g in Spain, which represents the highest level among the reported values, with a particularly high ratio (74%) of contamination by UV-320. There may be a specific contamination route for UV-320. Furthermore, the composition of contaminants differs significantly from that of Korea, where UV-328 accounted for 60% of the level, followed by UV-P. In Japan, UV-328 was rarely used, but in Korea, UV-328 may have been used as a substitute for UV-320. China had a total level of 84.2 ng/g for the four chemicals, although there was no data for UV-320. The level of UV-327 was higher in China than in Japan, but UV-326 and UV-P were at similar levels. The reason for the higher levels of Japanese mothers as compared to those in other Asian countries may be attributed to be related to the historical production and utilization of UV-320 and UV-327. The details of foreign regulations on BUVAs are unknown, and we speculate that UV-328 and UV-P are commonly used in Korea and Vietnam. Currently, exposure to UV-320 and UV-327 is still being observed. Therefore, continuous monitoring is necessary in Japan.

Comparisons of Primiparas and Multiparas Figures 3 and 4 compare the composition ratios of BUVAs and the average level of each compound in the breast milk of primiparas and multiparas. Multiparas are women who had 2 to 4 births. The mean total BUVA level in multiparas (233 ng/g lipid wt.) was 2.1 times higher than that in primiparas (112 ng/g lipid wt.). The composition of BUVAs in the breast milk of primiparas was 52% UV-327, followed by 12% UV-326 and UV-329. Multiparas had 42% UV-320, which was 4.7 times higher than that in primiparas (Fig. 3). There were several donors with extremely high levels of UV-320 in multiparas. UV-320 ($p<0.05$) was significantly higher in multiparas than in primiparas (Fig. 4). However, no significant difference in the levels of UV-326, -327, -328, -329, and -P between multiparas and primiparas ($p>0.05$) were observed. Our research on multiparas with high BUVAs revealed a strikingly high ratio of UV-320. Due to the lack of knowledge about the sources of these exposures, it is important to investigate the sources of contamination, such as food and water. UV-327 was also observed in high levels in multiparas. We investigated why the levels of total BUVAs were higher in multiparas than in primiparas. In general, the levels of organohalogen compounds, such as polychlorinated biphenyls, were higher in primiparas

Table 2. Levels (ng/g, lipid wt.) and Frequency (%) of Detection for the Six BUVAs in the Breast Milk Sample (N = 36)

	Mean	Max	Min	Median	95% CI[a]	Frequency
UV-320	78.3	525	3.21	17.2	32.2, 124	100
UV-326	32.3	150	0.654	24.9	21.5, 43.1	100
UV-327	42.2	288	< LOQ [b, c]	9.88	19.2, 65.1	75
UV-328	9.15	38.0	< LOQ [b, c]	5.60	6.07, 12.2	97
UV-329	27.6	162	0.748	18.6	16.7, 38.6	100
UV-P	26.6	237	< LOQ [b, c]	10.5	12.4, 40.8	89
ΣBUVAs	216	803	11.0	119	142, 290	-

[a] CI: confidence interval
[b] LOQ: Limit of quantitation. UV-327, 0.060 ng/g, lipid weight; UV-328, 0.010 ng/g, lipid weight; UV-P, 0.090 ng/g, lipid weight.
[c] LOQ was defined as 10 times (S/N=10) the noise level.

Table 3. Estimated Daily Intakes (ng/kg/day) of BUVAs via the Consumption of Breast Milk in Japanese Infant

	UV-320	UV-326	UV-327	UV-328	UV-329	UV-P	ΣBUVAs
Mean[a]	487	124	183	36.7	139	103	1,070
SD	942	72.2	251	26.0	159	125	1,260
Min	13.3	2.84	n.d.[b]	n.d.[b]	3.99	n.d.[b]	76.5
Max	3,060	259	866	94.0	582	513	4,410

[a] Calculation is based on an average milk intake rate of 1,000 mL.
[b] n.d.: not detected

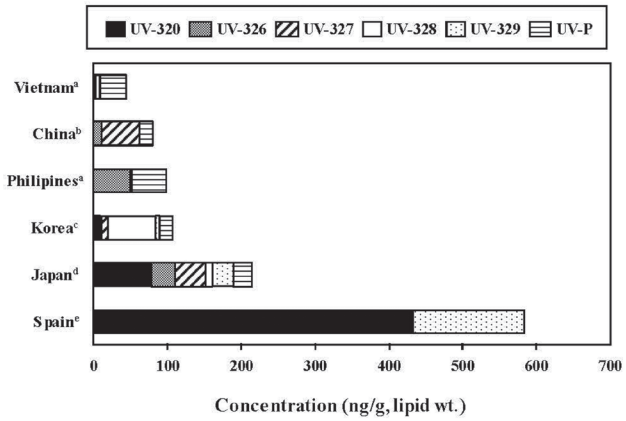

Fig. 2. Comparison of Mean Levels (ng/g, lipid wt.) for UV-320, -326, -327, -328, -329, and -P in Breast Milk Collected from Japan with Those Reported in Previous Studies.

[a]Kim *et al*. (2019),[6]
[b]Sun *et al*. (2022),[7]
[c]Lee et al. (2015),[8]
[d]this study
[e]Molins-Delgado *et al*. (2018)[9]

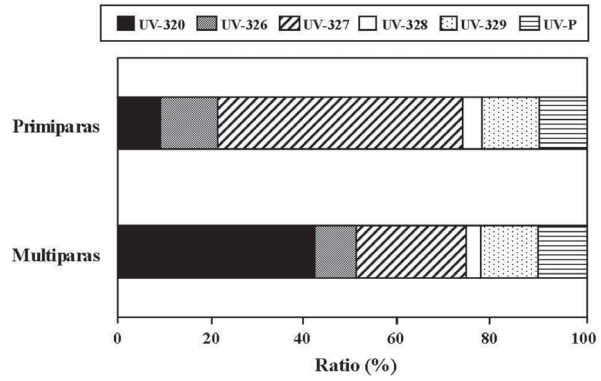

Fig. 3. The Average Contribution (%) of UV-320, -326, -327, -328, -329, and -P to the Total Level in Breast Milk Collected from Primiparas and Multiparas.

Multiparas are women with 2 to 4 births. The limits of quantitation for UV-320, -326, -327, 328, 329, and -P are 0.040, 0.050, 0.060, 0.010, 0.110, and 0.090 ng/g lipid wt., respectively.

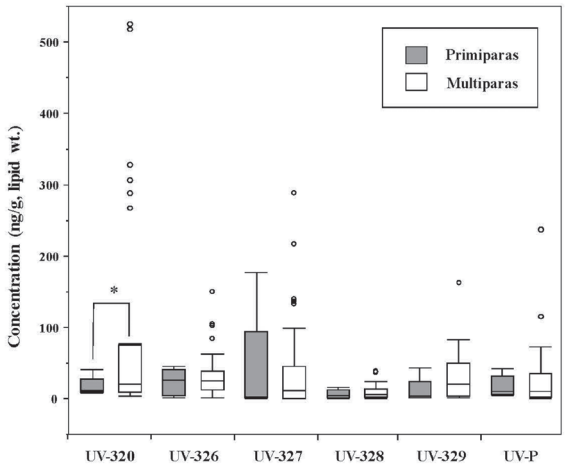

Fig. 4. Comparison of BUVA Levels in Breast Milk Collected from Primiparas and Multiparas

Multiparas are women with 2 to 4 births. Data are presented as box-and-whisker plots. The limits of quantitation for UV-320, -326, -327, 328, 329, and -P are 0.040, 0.050, 0.060, 0.010, 0.110, and 0.090 ng/g lipid wt., respectively. Open circles are outliers. *p < 0.05

than in multiparas.[10-15] Conversely, hexachlorobenzene[13], *tris*-4-chlorophenyl-methane[14], polybrominated diphenyl ethers[15], and hexabromocyclododecanes[15] were higher in multiparas than in primiparas. As described above, the levels of chemicals in breast milk differ due to differences in exposure amount, excretion, and metabolic rate. With regard to BUVAs, it is thought that the metabolism and daily intake of BUVAs are also affected, and it is assumed that the main source of exposure is food. Further studies using various foodstuffs are needed to clarify the variation in human exposure, accumulation levels, and exposure sources of BUVAs.

Estimation of BUVAs Intake by Infants through Lactation The data on BUVA contamination in breast milk samples showed that humans are constantly in contact with BUVAs. Because infants are particularly vulnerable, we used our results to estimate their BUVA intake from breast milk (Table 3).

A 3-month-old infant consumes 900–1,000 mL of breast milk/day, and BUVAs of 11.0–803 ng/g lipid wt. (0.459–26.5 ng/mL) were detected in the breast milk sample. Assuming that an infant weighing 6 kg breastfeeds 1,000 mL/day, it was estimated that they ingest approximately 76.5–4,410 ng/kg/day of BUVAs. To the best of our knowledge, there is a lack of toxicological data on BUVAs for use in the tolerable daily intake calculations. Therefore, the long-term adverse effects of the estimated intake are unpredictable.

From the above results, it was inferred that humans are exposed to BUVAs in their daily lives, highlighting the need for detailed toxicity studies on unregulated BUVAs. Further studies are necessary to help develop regulations for these chemicals in our living environment and prevent harmful exposure.

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Conflict of interest The authors declare no conflict of interest.

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