Photo-Conversion of Phenytoin to Ecotoxicological Substance Benzophenone
by Ultraviolet Light Irradiation in Aqueous Media

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Abstract
To determine the fate and behavior of pharmaceuticals in the aquatic environment, in this study, we investigated the photo-conversion of phenytoin (PH), which is one of the antiepilepsy drugs, induced by ultraviolet light (UV) irradiation in the aqueous media. The degradation of PH and the generation of its photoproducts were monitored by means of high-performance liquid chromatography (HPLC). Based on the fragmentation patterns in electrospray ionization time-of-flight mass spectrometry (ESI-TOF/MS/MS) and coupling patterns in nuclear magnetic resonance (NMR) spectrum, the main photoproduct of PH was determined as benzophenone. Evaluation of toxicity by means of luminescent bacteria test (ISO11348) indicated that a PH solution was nontoxic, whereas a UV-irradiated PH solution and a benzophenone solution were toxic (EC50: 19.46 mg/L and 24.40 mg/L). It was shown that benzophenone has a contribution to the increase of ecotoxicity of PH solution for luminescent bacteria after UV irradiation. This is the first report of the photo-conversion of PH to benzophenone induced by UV irradiation in the absence of photo-catalysts. These results indicate that the importance of evaluating not only parent compounds but also its photoproducts for the risk assessment of pharmaceuticals in the aquatic environment.

Keywords: Phenytoin; Photo-conversion; Aqueous media; ESI-TOF/MS/MS; NMR

1. Introduction
Pharmaceuticals are useful compounds for the purpose of diagnosis, prevention and therapeutics for the illness all over the world. In recent years, pharmaceuticals have been detected from the aquatic environment, including surface water, ground water and drinking water, at levels in the range of ng/L to µg/L on account of its discharge as intact or modified forms after having been administered to human and livestock [1-5]. Pharmaceuticals in the aquatic environment are persistence in water, absorbed to some substances such as sediments or taken up by the aquatic species. There are various factors having an influence on the persistence and fate of them. Processing at sewage treatment plants (STPs) such as membrane bioreactor, activated sludge processes and ultraviolet light (UV) treatment are effective for the purification of their sewage water. These methods are also able to remove various pharmaceuticals based on the biodegradation and the photodegradation [6,7]. Other reports indicate that pharmaceuticals can be removed from the aquatic environment in various pathways, including sorption, hydrolysis, biodegradation and photodegradation by sunlight [8-11]. However, STPs have been not completely equipped in the world. For example, the value of sewage coverage in Japan is 78.8% in 2018, especially low in local area. Excreted pharmaceuticals from human and livestock via urine may be flowed into and persisted in the aquatic environment without purification in the area where STPs are not fully equipped. Furthermore, the removal of pharmaceuticals by processing at STPs is not perfect and...
residual pharmaceuticals have been detected from the effluents from the STPs [12,13]. Considering the removal in the environment, the efficiency of sorption of pharmaceuticals to sediment is site specific due to its nature [14], and some pharmaceuticals have been designed to resist to biodegradation [15]. Sunlight is not also able to remove all pharmaceuticals in the aquatic environment [9], indicating that they are not necessarily inactivated or removed completely, and some of them persist in the aquatic environment.

The influence of pharmaceuticals, not only their persistence in the aquatic environment, has become an important issue. For the evaluation of the ecotoxicological effects, various ecotoxicological tests using aquatic organisms, such as Daphnia magna, Oryzias latipes and Brachinus calyciflorus, have been used and shown the toxicological potencies of pharmaceuticals for them [16-18]. In the view of genotoxic effects, the SOS chromotest and Ames test have been utilized [19,20]. These tests showed that pharmaceuticals discharged into the aquatic environment may act as the toxicological potencies for the aquatic species. It is reported that steroid hormones such as estrogen and 17β-ethinylestradiol flowed into the aquatic species. It is reported that steroid hormones such as estrogen and 17β-ethinylestradiol flowed into the aquatic environment showed the endocrine disruption action and induced the abnormality of a sexual organ [21-23]. Other pharmaceuticals may have biological activities to the aquatic species, but it is still unclear. The risk assessment of pharmaceuticals for not only human and livestock but also the environmental system is an important issue for the save of them and more research is required. Moreover, it is possible that the various factors such as hydrolysis and photo-irradiation may have an influence on the biological activities of pharmaceuticals for the aquatic organisms.

Especially, the effect of UV irradiation is significant. UV irradiation induces the change of the chemical structure of the pharmaceutical, and probably the nature of it. Our previous reports showed the photo-isomerization of sulindac induced by UV irradiation [24]. It was reported UV irradiation induced the photo-conversion and increased or decreased the ecotoxicological effects of pharmaceuticals [25,26], as the result of the photodegradation which induced the decrease of the parent compound or the generation of photoproducts which may have more toxicological potent compared to the parent compound via photo-conversion from original compounds. There are a lot of pharmaceuticals and their chemical structures are various, so the photochemical reaction of them are also various. Many reports have been published focused on the photoproduct formation from several pharmaceuticals in response to UV irradiation, although the influence of these reaction on ecotoxicity to the aquatic organisms has generally not been examined.

From these background, in this study, the behavior of antiepilepsy drug phenytoin (PH) in the aquatic environment was focused. It is reported that PH has been detected from the aquatic environment [12,13], and degraded by UV irradiation [27-29]. On the other hand, there are no reports determining the chemical structure of PH photoproduct in the absence of photo-catalyst, which may be generated in the aquatic environment. Furthermore, the influence of UV irradiation on the ecotoxicological effects of PH on the aquatic organisms is unclear.

The aim of this research is to investigate the photo-conversion of PH, determine the chemical structure of a photoproduct and evaluate its ecotoxicity to the aquatic organisms. In this work, photodegradation of PH exposed to UV irradiation at 254, 302 and 365 nm was demonstrated, and the degradation rate was monitored by means of high-performance liquid chromatography (HPLC). Identification of the chemical structure of its photoproduct was carried out using electrospray ionization time-of-flight mass spectrometry (ESI-TOF/MS/MS) and nuclear magnetic resonance (NMR), and the ecotoxicity of PH and its photoproduct was examined by employing the luminescent bacteria test, ISO11348.

2. Experimental

2.1. Chemicals

All of reagents and organic solvents were of special grade or HPLC grade. Methanol, ethanol, hydrochloric acid, sodium chloride, dimethyl sulfoxide, diethyl ether and acetic acid were purchased from Tokyo Chemical Industries (Tokyo, Japan). Methanol-α-d4 was purchased from Merck (Darmstadt, Germany). Milli-Q (18.2 Ω/cm) water was prepared by using a Milli-Q water purification system (Merck).

2.2. UV spectral analysis

All test compounds were dissolved in ethanol at the final concentration of 10-100 µmol/L. UV absorption spectra were recorded with a UV2450 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) interfaced to a PC for data processing. The absorption-maximum wavelength (λmax, nm) was obtained from these results. The molar absorbance coefficient (ε) of each compound was calculated from the absorbance at λmax.

2.3. Photodegradation experiments

UV irradiation was carried out in the light cabinets 20W FL20S BLB black light lamp (Toshiba, Tokyo, Japan), 6W UVM-57 Handheld UV lamp (UVP, Upland, CA, USA) and 20W GL-20 UV lamp (Toshiba) equipped with electrical discharge tube and special filters emitting light at 365 nm (UV-A), 302 nm (UV-B) and 254 nm (UV-C) (Irradiation
**Chromatography**

Intensity was as follows: UV-A: 1800 µW/cm²/s, UV-B: 1250 µW/cm²/s, UV-C: 150 µW/cm²/s at 15 cm from the source. Irradiation intensity was measured using a digital radiometer with 365, 302 and 254 nm sensor (UVX-36, UVX-31 and UVX-25, UVP). PH was initially dissolved in methanol (1 mg/mL) and this solution was diluted by the addition of Milli-Q water for the preparation of a test sample (PH 1 µg/mL) in a glass vessel. This sample was irradiated by the UV lamp (water depth was 3 cm, distanced from lamp source about 15 cm). Irradiation times were 0, 1, 2, 4, 6, 12, 18 and 24 hr at 20 °C. Control samples were also prepared for the same condition but covered with the aluminum foil to interrupt UV irradiation. Irradiated samples by UV were condensed by the solid phase extraction using the cartridge (Oasis HLB cartridge 3cc, Waters Corporation Milford, MA, USA) in the multi-sample concentration system (Varian Sample Preparation Vac Elute SPS 24, Agilent Technologies, Santa Clara, CA, USA). Samples were eluted using methanol (2 mL), and concentrated under the reduced pressure. Residual substances were dissolved in methanol (500 µL) following to the HPLC analysis. Also, a main PH photoproduct was isolated using HPLC followed by the structure determination.

2.4. HPLC analysis

The decrease of PH and generation of photoproducts were monitored with an HPLC (L-7110, HITACHI, Tokyo, Japan) system equipped with an Inertsil ODS-3 column (5 µm, 4.6 x 150 mm, GL Sciences Inc., Tokyo, Japan), a UV detector (L-7440, HITACHI), and a Chromato-Integrator (D-2500, HITACHI). The UV detector was set at 254 nm. A mixture of Milli-Q water and methanol (1:1) containing acetic acid (0.1%, v/v) was used as an isotropic mobile phase at a flow rate of 0.5 mL/min. Retention times were 20.9 min (PH) and 72.2 min (PH photoproduct). After the structure determination, the HPLC analysis of benzophenone was performed with the same conditions as in PH (Fig. S1).

2.5. Structure determination of PH photoproduct

Identification of the PH photoproduct was carried out using ESI-TOF/MS/MS (Qstar® XL, Applied Biosystems, Waltham, MA, USA) and NMR (Brucker AVANCE600, Brucker, Billerica, MA, USA). One main PH photoproduct was isolated from the photo-exposed PH solution by means of HPLC and injected into the ESI-TOF/MS/MS by NanoSpray Infusion. Analysis was conducted in the positive ion mode. Calibration was carried out with p-aminophenol (1 nmol/mL) and reserpine (1 nmol/mL) before analysis. Source/gas parameters of TOF/MS/MS were as follows: ion source gas 1.20 pound-force per square inch (psi), ion source gas 2.0 psi, CurtainGas™: 25 psi, ionspray voltage (positive) : 5500 V. TOF/MS/MS parameters were as follows: declustering potential : 60.0 V, focusing potential : 280.0 V, declustering potential 2 : 10.0 V, collision energy : 20.0 V, collision gas : 5.0. Scan type was both TOFMS mode, which is used for measuring the MS, and Product Ion Scan mode, which is used for measuring the MS/MS. Also, a PH photoproduct was fractionated using HPLC repeatedly, and extracted with diethyl ether-1N hydrochloric acid (1:1, 4 mL). After centrifugation (3000 rpm, 5 min), a diethyl ether layer was concentrated and the extracted PH photoproduct was dissolved in methanol-d4 following to the NMR analysis. Instrumental analysis such as HPLC, ESI-TOF/MS/MS and NMR of benzophenone was performed with the same methods as in the UV-irradiated PH. Results of ESI-TOF/MS/MS and NMR analysis of benzophenone were shown in supplemental figures (Fig. S 2-3).

2.6. Luminescent bacteria test

Luminescent bacteria test was carried out following ISO11348. This method is based on measuring the light emission decrease of the luminescent bacteria (Photobacterium phosphoreum) which relates to the amount of the toxic stress. Measurements of luminescence were carried out using the multilabel counter (ARVO™ MX, Perkin Elmer, Waltham, MA, USA). Toxic effects were expressed as the median effective concentration (EC₅₀, mg/L). A sample preparation method is as follows; PH (1 mg) was initially dissolved in dimethyl sulfoxide (DMSO, 50 µL) and then diluted further in Milli-Q water (950 µL). Then 22 % sodium chloride solution (NaCl sol., 100 µL) was added in order to make NaCl concentration of the sample 2 %. This sample was diluted to 2, 4, 8, 16, 32, 64 and 128 times using a 2 % NaCl sol. and used. A control sample which did not contain PH was also prepared. A photo-exposed sample was prepared as follows; PH in methanol (1 mg/mL, 1 mL) was added in Milli-Q water and irradiated UV (PH was irradiated by UV-C for 24 hr). After the irradiation, the sample was concentrated 100 times by the solid phase extraction. A residual substance was prepared for the luminescent bacteria test sample by the same manner as in PH. Measurements of the light emission were made after 15 min of exposure using the multilabel counter and compared to the control sample. Toxicity of the sample was evaluated by calculating the decrease of the light emission compared to the control sample.

2.7. Statistical analysis

Results of the luminescent bacteria test (EC₅₀) were expressed as mean ± standard deviation (S.D.). EC₅₀ was calculated using the Probit analysis (Ecotox Statics ver. 2.3; The Japanese Society of Environmental Toxicology).
3. Results and discussion

3.1. Photodegradation of PH by UV irradiation

The fate and behavior of pharmaceuticals in the aquatic environment should be concerned for saving the aquatic ecosystems. In this work, the photo-conversion of PH, which is one of the antiepilepsy drug, induced by UV irradiation was investigated. The chemical structure of PH is shown in Fig. 1. The photodegradation of PH and generation of PH photoproduct by UV irradiation were monitored by means of HPLC. HPLC chromatograms of a PH solution before and after UV-C irradiation for 12 hr are shown in Fig. 2. Furthermore, the amounts of PH and its photoproduct evaluated by HPLC are shown in Fig. 3, those vertical axis showed the residual rate of PH or PH photoproduct for the amount of PH before UV-C irradiation, and those horizontal axis showed the irradiation time of UV-C. PH showed dissipation of the peak area and the appearance of new peaks indicated the generation of photoproducts in HPLC analysis after the exposure to UV-C for 12 hr (Fig. 2). Multiple photoproducts of PH were generated by UV-C irradiation, and the generation of one main photoproduct was remarkable compared with others (Retention times were 20.9 min (PH) and 72.2 min (the main photoproduct of PH)). It is expected that the main photoproduct of PH has lower polarity than PH because the photoproduct was eluted slower than PH. Results of the UV irradiation experiment indicate that PH is degraded upon the exposure to UV irradiation at 254 nm with the generation of the photoproducts, although PH was not degraded by UV irradiation at 302 and 365 nm (data not shown). It is well known that pharmaceuticals are photodegraded faster at shorter UV wavelength, this is as expected, since the energy of the radiation is higher at shorter wavelength. This is also in agreement with the reported findings [30]. PH was degraded to less than 40% after UV-C irradiation for 2 hr, and completely degraded after 6 hr (Fig. 3). However, the main photoproduct of PH amounted to more than 200% after UV-C irradiation for 4 hr indicating the molar absorbance coefficient of the PH photoproduct is bigger than PH. The PH photoproduct was not degraded by UV-C irradiation for 24 hr. PH was degraded completely but the photoproduct was not degraded during UV-C irradiation for 24 hr (Fig. 2), and there was no indication that a further photoproduct was generated from the initially formed photoproduct (Fig. 3). On the other hand, the control was unchanged indicating that the degradation of PH was not due to the other factors, such as hydrolysis and temperature.
3.2. Structure determination of the photoproduct

Structure determination of the main PH photoproduct was carried out by the instrumental analysis such as ESI-TOF/MS/MS and NMR. In the ESI-TOF/MS/MS analysis, the mass spectrum showed the protonated ion \([\text{M} + \text{H}]^+\) and sodium-adducted ion \([\text{M} + \text{Na}]^+\) at \(m/z\) 183.0809 and 205.0752 (Fig. 4A). Fragment ion peak of the protonated ion peak of the photoproduct was detected at \(m/z\) 183.1005 (Fig. 4B). Fragment ion peak at \(m/z\) 105.0360 (Fig. 4B). Fragment ion peak at \(m/z\) 105 corresponds to the benzyl group and characteristic loss of the phenyl group, indicating that the photoproduct of PH is benzophenone. The chemical structure of benzophenone is shown in Fig. 5A. The molecular weight of benzophenone is 182.22 g/mol and the value of \(m/z\) of the protonated benzophenone is 183, which is corresponded with the results of the ESI-TOF/MS/MS analysis. Furthermore, from the numbers of proton and carbon in the chemical structure and the chemical shift of the results in \(^1\text{H}-\text{NMR}\) and \(^{13}\text{C}-\text{NMR}\) analysis, it is also tempting to speculate that the photoproduct of PH is benzophenone (Fig. 5B). These results suggest that PH is photo-converted to photoproducts, one of which is benzophenone, by UV irradiation. Results of HPLC, ESI-TOF/MS/MS and NMR analysis using benzophenone standard were the same as that of the UV-irradiated PH (Fig. A.1-3). In the UV spectrum analysis, the molar absorbance coefficient and absorption-maximum wavelength of benzophenone are 15,540 and 256 nm, respectively, which are bigger than those of PH (1,021 and 252 nm) (data not shown), having a contribution for the generation rate of benzophenone which is above the residual rate of PH in the HPLC analysis (Fig. 3).

The determination of the chemical structure of photoproducts may provide the information of photo-conversion mechanism. In this study, detailed photochemical reaction mechanism is not evaluated, but it is tempting to speculate that the UV irradiation induces the opening of hydantoin ring of PH via the generation of radical species, followed by the oxidation and decomposition to form the benzophenone finally. From the results of the UV irradiation experiments, benzophenone is photo-stable in this experiment and not degraded after UV irradiation at 254 nm for 24 hr (data not shown) which is corresponding to the result as shown in Fig. 3, which indicating that PH was degraded completely but its
photoprodut was not degraded. Photo-conversion of parent compounds to their photoproducts is occurred when the chemical bonds compose the compounds in the ground state absorb light energy and forming an excited state that triggers a photochemical reaction such as bond cleavage and addition. Various photochemical reactions may be induced by UV irradiation [31-33]. Moreover, the presence of photo-catalysts, which serve as the energy supplier, makes the photochemical reaction mechanistically more complex on account of the photo-generated hydroxyl and other radicals. They are involved in the chemical reaction followed by the generation of a lot of its photoproducts. It is reported that various photoproducts are generated by UV irradiation in the presence of some photo-catalysts [34]. The variety of photoproducts generated by the photo-conversion is dependent on many factors, such as the wavelength of UV exposure, chemical structure, the kinds of functional groups and the presence of photo-catalysts and other chemical substances.

Conversion of PH to benzophenone by UV irradiation with photo-catalysts has been reported previously [27]. However, this is the first report exterminating the chemical structure of PH photoprodut generated by UV irradiation in the absence of photo-catalysts. This photo-conversion may occur in the aquatic environment, where there are no photo-catalysts. For example, various pharmaceuticals have been found to be present in effluents from STPs, where water treatment included primary settling and an activated sludge process, and appeared to be photodegraded on exposure to UV irradiation for sterilization. PH has been detected in STPs effluents in the range of ng/L to µg/L concentration [12,13], so residual PH from sludge systems may be transformed into the photoprodut by UV irradiation during the sterilization treatment. Also, it has been reported that benzophenone was detected from the aquatic environment including surface water, sediment and urban sewage [35,36]. It is unclear that the photo-conversion from PH to benzophenone induced by UV irradiation during the sewage treatment has a contribution to the presence of benzophenone in the aquatic environment, but this study suggests that the behavior of PH in the aquatic environment should be concerned because of the ecotoxicological effects of its photoprodut on the aquatic organisms.

3.3. Toxicity evaluation using the luminescent bacteria test

Influence of a PH solution and a UV-irradiated PH solution on aquatic organisms was investigated by means of the luminescent bacteria test. A PH solution had no effect, and so the EC50 of a PH solution could not be calculated (Table 1). In contrast, a UV-irradiated PH solution showed marked toxicity (EC50: 19.46 mg/L). Benzophenone also showed toxicity in this test (EC50: 24.40 mg/L).

The results of the luminescent bacteria test, which is utilized to evaluate the ecotoxicological effects of test compounds on the aquatic organisms, indicate that PH would not be toxic to aquatic organisms but a UV-irradiated PH and its photoprodut benzophenone were toxic (Table 1). It is indicated that the photoprodut contributes to the increase of toxicity to the luminescent bacteria. Toxicalogical studies have shown that benzophenone is regarded as the third-generation environmental pollutant (endocrine disruptors) and its potential high risk to ecosystem and human health over a long time period should be made widely known because of its biological toxicity effect [37]. Moreover, there are some reports that benzophenone showed ecotoxicological toxicities as a result of in vitro test including the luminescent bacteria toxicity, genotoxicity and hormonal activity [38] similarly to our study. These results suggest the importance to investigate the ecotoxicological effects of the photoproduts, which may be generated from the pharmaceuticals discharged into the aquatic environment by the photo-irradiation. The concentration range of both UV-irradiated PH and benzophenone are mg/L determined in luminescent bacteria test, which is higher than the concentrations at which PH has been detected in aquatic environments (µg/L-ng/L). Although the dissociation of the toxic concentration and the detected concentration, the results of ecotoxicological test should not be underestimated because it is unclear the effects of chronic exposure, mixture exposure and acute toxicity utilized other aquatic species. In future work, it will be important to evaluate the toxicological potencies of the pharmaceuticals and their photoproduts using the several tests to clarify the ecotoxicity of them from the multiple viewpoints.

There are several reports indicating that the toxicity of pharmaceuticals to aquatic organisms can be altered by UV irradiation on account of the formation of the photoprodut. It is reported that generated photoproducts by UV irradiation have a contribution to the change of toxicological effects [25,26]. In the case of diuretic drug furosemide, its photoprodut is dimer [39] and has mutagenic potential [40]. Our previous reports also indicate the change of toxicological effects of some pharmaceuticals induced by UV irradiation [41,42]. These reports

| Compounds      | EC50 (mg/L) |
|---------------|------------|
| PH            | N.D.       |
| UV-irradiated PH | 19.46 ± 8.72 |
| benzophenone   | 24.40 ± 9.36 |

n=3-4, values in mean ± S.D.; N.D., no detectable toxicity.
re-emphasize that it is important to evaluate the effects of UV irradiation on the ecotoxicity of pharmaceuticals in aquatic environments. It is important to consider the UV irradiation as the major factor for the fate and behavior of the pharmaceuticals in the aquatic environment on account of its influence on the residual rate, photo-conversion and the ecotoxicological potencies, and it is needed to proceed the further research due to the photochemical behavior of many pharmaceuticals have been still unclear.

4. Conclusion

In conclusion, we have shown that one of the photoproduct of PH generated by UV irradiation is benzophenone, which has a contribution to the increase of photoproduct of PH generated by UV irradiation is the further research due to the photochemical behavior of the ecotoxicological potencies, and it is needed to proceed the further research due to the photochemical behavior of the pharmaceuticals persist in the aquatic environment. Further study is needed to clarify the fate and behavior of the pharmaceuticals for the protection of the aquatic ecosystems.

Supporting information

HPLC chromatogram, ESI-mass spectrum and 13C- and 1H-NMR spectroscopic data of benzophenone are available via the WEB at http://chromsoc.jp/Journal/SI.html.

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