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Determination of free radicals generated from light exposed ketoprofen

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Abstract

Ketoprofen [(RS)-2-(3-benzoylphenyl)propanoic acid] has been widely used for the treatment of inflammatory diseases and musculoskeletal injury. However, there is concern over its phototoxicity and photosensitization potential as side effects. In the present study, we examined photodynamic action of ketoprofen, determined free radicals and active oxygen species generated from ketoprofen by photo-irradiation, which have been implicated as the phototoxicity and photosensitization. The generation of superoxide anion, hydroxyl radical and singlet oxygen were determined by electron spin resonance (ESR), chemiluminescence analyzer (CLA) and the on-line photo irradiated superoxide anion detection system, when ketoprofen was exposed by the ultraviolet lamp or the chemical lamp. The on-line light exposed superoxide anion detection system was developed to catch superoxide anions generated at an instant and estimate quantitatively risk of the light-sensitive disease of drugs. These radicals were maintained for 20 min at the ESR spectrum measurement using the spin trapping reagent, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), though these signals were declining gradually. The degradation products were analyzed to identify the chemical structures. The pathway of the photo degradation and generation of the active oxygen species was estimated by the experimental results obtained in this study.
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Keywords: Free radicals, Active oxygen species, Superoxide anion, Light-sensitive drugs, Electron spin resonance, Chemiluminescence analyzer

1. Introduction

Ketoprofen [(RS)-2-(3-benzoylphenyl)propanoic acid] has been widely used for the treatment of inflammatory diseases and musculoskeletal injury. However, there is concern over its photosensitivity potential as adverse effects. A lot of cases of the photosensitivity attributed to ketoprofen, such as erythema, edema and pigmentation, were reported. The photosensitivity is classified into phototoxicity [1–4] and photoallergy [2,5]. The phototoxicity is not related to the immunity response, everyone has possibility to be suffered by the symptom if exposed by the sun after taking the ascribed substance. On the other hand, the photo allergy is caused due to the immunity response, and the symptom is not relative to the amount of the substance and the energy of the sunlight. It is considered that the phototoxicity and photo allergy are generally correlated to each other: free radicals generated from the substance having the chemical structure to be easily excited by the photo energy attack the human cells (phototoxicity) and bond to neighbor proteins so that the antigen is grown (photo allergy) [5]. The mechanism of the photosensitivity by ketoprofen has not been obviously revealed, although the free radicals are implicated as these phototoxicity and photo allergy [5–15]. The objective of this study was to determine the active oxygen species generated from ketoprofen, and estimate the degradation pathway and the mechanism of generation of the active oxygen species, when ketoprofen was exposed by the sunlight. The results of this study were expected to contribute to the development of new excipients in ketoprofen dosage form to prevent or decline the phototoxicity. We successfully determined the superoxide and hydroxyl radical by ESR and singlet oxygen by the CLA [16]. Quantitative estimation of generated superoxide anion

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was performed by the on-line photo irradiated superoxide anion detection system that was developed to catch superoxide anions at an instant and estimate quantitatively risk of the light-sensitive disease of drugs. The mechanism and pathway of the generation of these active oxygen species were consequently estimated according to the results of the above studies and the identification of photo degradation products.

2. Experimental section

2.1. Materials

Ketprophen was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Tetra-nitro methane (TNM) and (±)-epinephrine were purchased from Sigma-Aldrich (St. Louis MO). 5,5-Dimethyl-1-pyroline-*N*-oxide (DMPO) was obtained from Dojindo Laboratories (Kumamoto Japan). Acetonitrile and methanol of HPLC grade, ammonium acetate, dimethyl sulfoxide (DMSO), ethanol and ethyl acetate were purchased from Wako Chemical Industries (Osaka, Japan). N-hexane was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). Chloroform-d1 involving 0.03 vol% tetramethylsilane (TMS) for NMR was obtained from Merck Japan (Tokyo, Japan). Laboratory water was purified using a Milli-Q gradient A10 Elix with EDS polisher system water-purification (Millipore, Bedford, MA, USA).

2.2. Equipments

The light source was an ultraviolet lamp GL-6 (6 W) (maximum wave length 254 nm) (Panasonic, Tokyo, Japan), a chemical lamp FL6BL (6 W) and a white fluorescence lamp FL6W (6 W) (Toshiba, Tokyo, Japan). The emission spectra of the chemical lamp and the white fluorescence lamp are indicated in Fig. 1. ESR was JES-RE1X (JEOL, Tokyo, Japan) and CLA was CLA-FS1 (Tohoku Electron, Miyagi, Japan). An ultraviolet lamp of CLA was UV GL-25, MINERALLIGHT LAMP, MULTIBAND UV-254/366 nm (115 V, 60 Hz, 0.16 A) (UVP. INC, CA 91778, USA). On-line photo radical detection system consisted of Intelligent pumps; LC-10AD (Shimadzu, Kyoto, Japan) and L-6300 (Hitachi, Tokyo, Japan), a column oven; CTO-6A (Shimadzu, Kyoto, Japan) and a UV–vis detector; SPD-6AV (Shimadzu, Kyoto, Japan), a chemical lamp FL6BL (Tohoku Electron, Miyagi, Japan) and the Teflon PTFE tube was 2.5 m × 0.5 mm i.d. (Nishio, Tokyo, Japan). Mass Spectrometer was JMS600 (JEOL, Tokyo, Japan) and 1100 Series LC/MSD (Agilent). NMR was JNM-LA270 (JOEL, Tokyo, Japan).

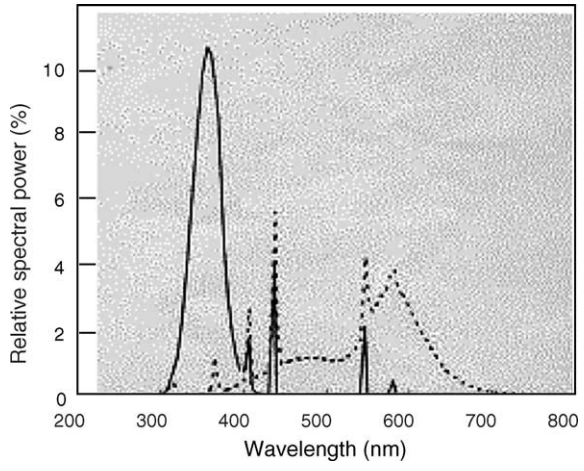

Fig. 1. Emission spectra of chemical lamp FL6BL and white fluorescence lamp FL6W: (—) chemical lamp, (···) white fluorescence lamp.

2.3. Determination of decomposition curve

Ketoprofen acetonitrile/water (1:1) solution (0.1 mM) was contained in a 1 cm × 1 cm quartz cell, exposed by each of the three lamps, an ultraviolet lamp GL-6, a chemical lamp FL6BL and a white fluorescence lamp FL6W. A volume of 10 μL of each exposed solution was injected into the HPLC system under the following conditions:

Mobile phase: acetonitrile/methanol/20 mM ammonium acetate (30:50:20), flow rate: 1.0 mL/min, column: GL-PACK Nucleosil 250 mm × 4.6 mm i.d., Column temperature: 40 °C, detection wavelength: 254 nm.

2.4. The off-line photo-irradiation/ESR experiments

Ketprophen (1 mM) in acetonitrile/water (1:1) was prepared as the sample solution. A volume of 5 μL of DMPO was added to each 0.3 mL of the sample solution and exposed by the ultraviolet lamp GL-6 for 0, 5, 10, 30, and 60 min, respectively. The same measurements were conducted for the sample solution without DMPO, and 5 μL of DMPO was added to the sample solutions after irradiation. These exposed sample solution of ketoprofen were applied to the HPLC analysis to monitor ketoprofen and the photo degradation products. The HPLC conditions were the same as that of the determination of decomposition curve of ketoprofen, except injection volume of 5 μL. A volume of 50 μL of DMSO, 50 μL of 25 mM NaOH, 5 μL of DMPO, 50 μL of 30% H_2O_2 and 50 μL of water were mixed to make the reference solution. ESR spectra of these solutions were obtained under the following conditions. Otherwise, the sample solution exposed for 60 min was transferred into an ESR sample tube and consecutively measured the ESR spectra at every 90 s. Measurement conditions: center field of 336.5 mT, sweep time of 5 s, sweep width of ±5 mT, field modulation width of 0.063, receiver gain of 500, time constant of 0.03 s.

2.5. Chemiluminescence analyzer

Ketoprofen powder was placed on the stainless and flat-bottomed sample container (10 mm × 50 mm i.d.) and irradiated by 254 and 366 nm of light beam for 5 s in air atmosphere, at room temperature. CL spectrum was obtained through a photo filter 330–700 nm.

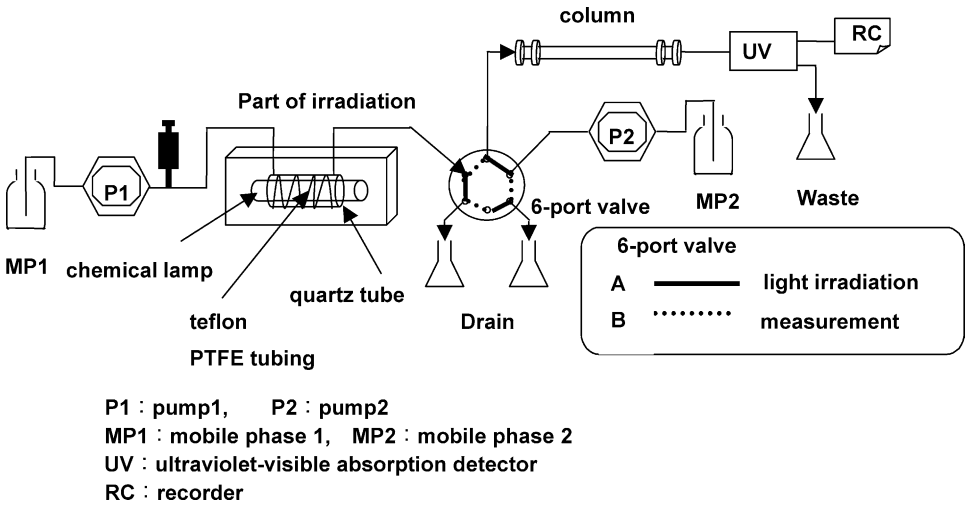

Fig. 2. Schematic diagram of on-line photochemical HPLC/UV system and operating configurations of column switching technique.

2.6. The on-line photo irradiated superoxide anion detection system preparation of the sample solutions

Ketprophen (10 mM) and DMSO (10 mM) in acetonitrile/water (1:1) were respectively prepared as the sample solution. TNM (1 μM) in acetonitrile/water (1:1) was prepared as reagent solutions.

2.7. Equipments

The column-switching on-line HPLC system, as depicted in Fig. 2, equipped with a light exposing cell, a six-port switching valve, a reagent-flow pump, an analytical HPLC and UV–vis detector. The light irradiation equipment was made up of a reaction coil and a chemical lamp FL6BL: 1.5 M of the Teflon tube was knitted and coiled on a tube of quartz (15 cm and 2.5 cm i.d.), and the chemical lamp was inserted into this quartz tube, to prevent deterioration of the Teflon tube by heat of lamp. This light exposing part was veiled with an aluminum-coated box (20 cm × 27 cm × 20 cm) to protect from the room light. A volume of 5 μL of 20 mM the sample solution was injected into the system and run together the reagent solution, carried by the pump 1. The pump 1 was running for 256 s at 0.1 mL/min, so that ketoprofen and TNM were exposed by UV for 177 s in the tefron tube. After the irradiation, the valve was switched from A to B to carry the light exposed solution into the analytical column by the pump 2. The valve was returned to the original position after flowing the mobile phase through the reaction coil for 1 min, intercepting the pump 2 flow from the reaction coil to protect the tefron tube against high pressure.

2.8. HPLC conditions

Mobile phase: acetonitrile/methanol/20 mM ammonium acetate (30:50:20), flow rate: 1.0 mL/min, column: GL-PACK Nucleosil 250 mm × 4.6 mm i.d., column temperature: 40 °C, detection wavelength: 366 nm.

2.9. Isolation and identification of the photo degradation products

2.9.1. TLC isolation of the photo degradation products

Ketoprofen's film on a laboratory dish was exposed by the ultraviolet light GL-6 for 24 h. The sample was dissolved with chloroform and spotted on a silica gel plate and separated by *n*-hexane/ethyl acetate (2:7). The bands absorbing 254 nm light were isolated and extracted with chloroform and/or methanol.

2.10. Mass spectrometry

The isolated sample was dissolved in a small amount of methanol, applied to the mass spectrometry. The mass spectra were obtained by EI mode MS and the direct API method under the following conditions. The EI mode MS: filament current of 300 μA, accelerating voltage of 3 kV, an electron energy of 70 V, an ion chamber temperature of 200 L, a probe temperature of 60–300 L and a rate of 128 L/min, the resolution of 500. The direct API method: Agilent 100 Series LC/MSD (positive mode).

2.11. NMR spectrometry

The isolated degradation compound was dissolved with chloroform-d1 in a sample tube of 5 mm i.d. ^{1}H NMR spectrum was obtained by 16 times accumulation and the chemical shifts were adjusted to 0 ppm of TMS.

3. Results

3.1. Photodecomposition of ketoprofen

Decomposition of ketoprofen and its degradation products was monitored by the peak area determination on HPLC

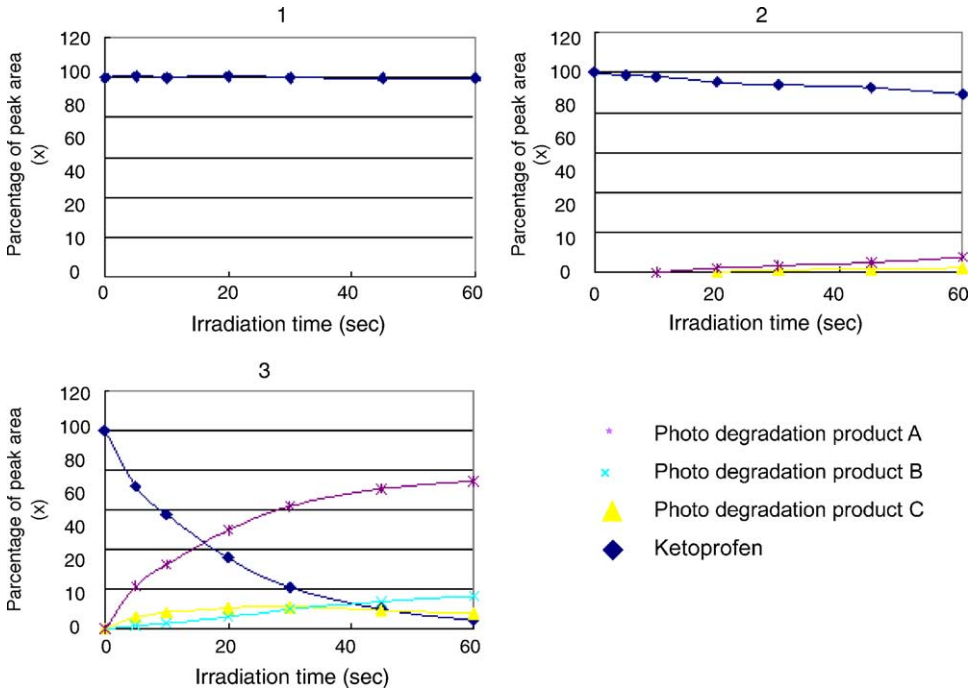

Fig. 3. The graphs of photo decomposition of ketoprofen and generation of its photo degradation products (1) white fluorescence lamp FL6W (6 W); (2) chemical lamp FL6BL (6 W); (3) ultraviolet lamp GL-6 (6 W).

to examine differences of degradation ways among the three lamps. The light energy was the same for the three lamps, which were 6 W. The plots of their peak ratio versus irradiation time are shown in Fig. 3. The degradation was not observed when exposed by the white fluorescence lamp FL6W. Ketoprofen was slightly or rapidly decomposed by the chemical lamp FL6BL or the ultraviolet lamp GL-6, respectively. The degradation products obtained using both lamps were the same three compounds. The chromatograms of the ultraviolet lamp GL-6 are shown in Fig. 4.

3.2. Detection of free radicals by the off-line photo-irradiation/ESR experiments

The chemical lamp and fluorescence lamp could not provide any clear signals of the free radicals due to the sensitivity of ESR spectrometer. We therefore used the ultraviolet lamp instead of the other two lamps in the ESR experiments. The ESR spectra of ketoprofen with DMPO, and DMSO with DMPO are shown in Figs. 5 and 6, respectively. ESR spectra of the solutions irradiated by ultraviolet for 5, 10, 30 and 60 min indicated generation of superoxide anion for ketoprofen and DMSO, and generation of hydroxyl radical for ketoprofen. When the trapping reagent (DMPO) was added to the sample solutions after irradiation, no signals were obtained in the ESR spectra. No any free radicals were observed in sole DMPO solution or ketoprofen and DMSO solutions without DMPO, even though irradiated by the 254 nm UV lamp. The signals of the ketoprofen solution were assigned comparing the spectrum of the reference solution. The spectrum of the reference solution clearly indicated the peaks of alkyl radical, hydroxyl radical and superoxide anion. These signals were detected in the exposed ketoprofen, although the intensities of their signals were extremely lower than ones of the reference solution. The ketprophen solution irradiated for 30 min gave the highest intensity of ESR signals. The rate of decomposition of ketoprofen in the exposed sample solution at each irradiation time is summarized in Table 1.

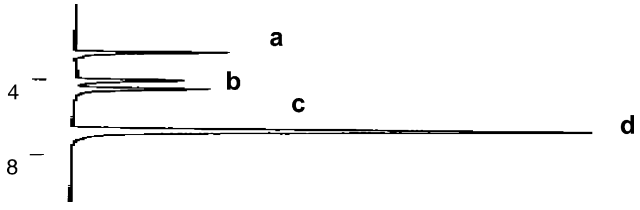

Fig. 4. Chromatogram of degradation products of ketoprofen by the ultraviolet lamp GL-6 Mobile phase: acetonitrile/methanol/20 mM ammonium acetate (30:50:20), flow rate: 1.0 mL/min, column: GL-PACK Nucleosil 250 mm × 4.6 mm i.d., column temperature: 40 °C, detection wavelength: 254 nm, injection volume: 10 μL; (a) ketoprofen, (b) degradation product C, (c) degradation product B, (d) degradation product A.

3.3. Detection of singlet oxygen by chemiluminescence analyser

The emission spectra of ketoprofen, irradiated by either 254 or 366 nm wavelength light, were measured using the chemiluminescence analyzer (CLA) [17]. The chemiluminescence was generated when the excited singlet oxygen return to the stable triplet oxygen. The chemiluminescence

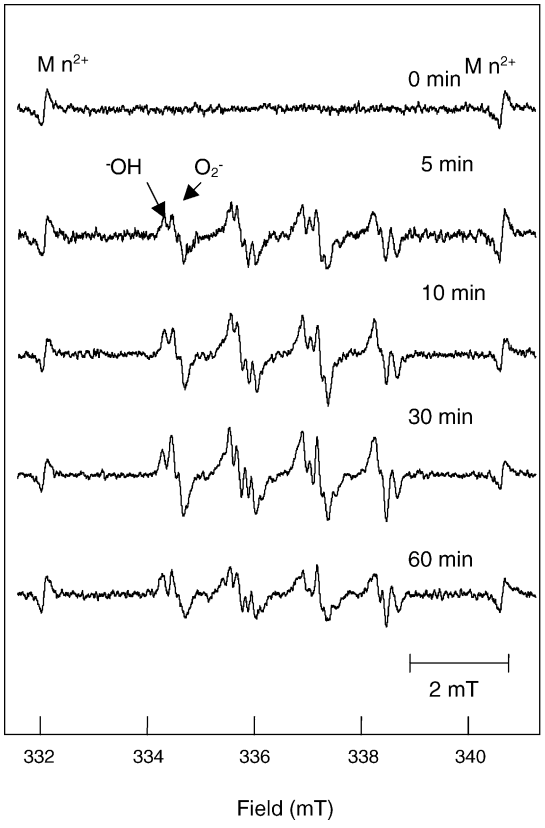

Fig. 5. ESR spectra of ketoprofen solution containing DMPO, exposed by ultra violet for several minutes.

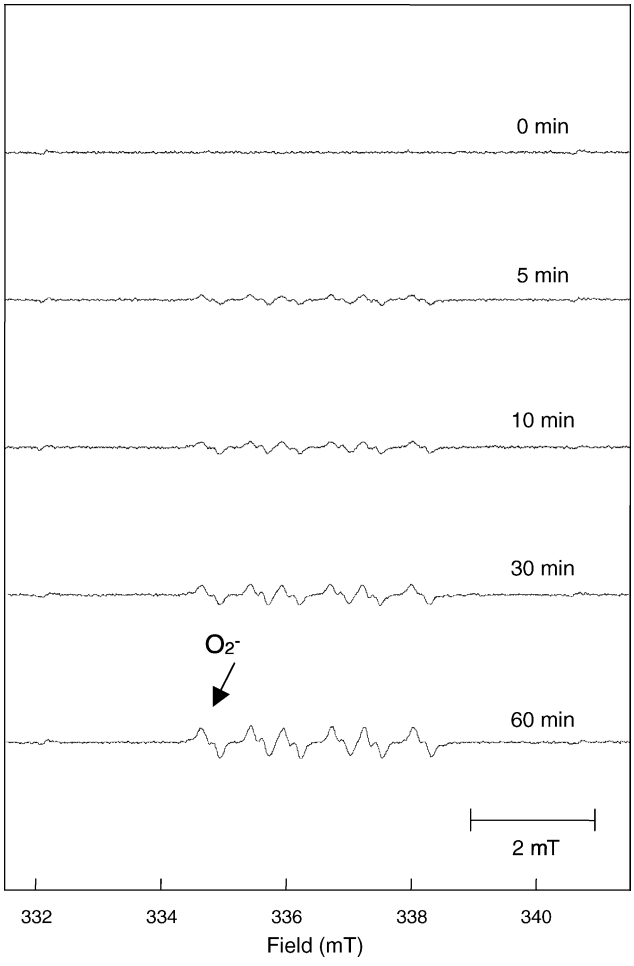

Fig. 6. ESR spectra of DMSO solution containing DMPO, exposed by ultra violet for several minutes.

spectra of ketoprofen are shown in Fig. 7. The slight differences of emission peaks' wavelength and intensity were indicated between the both spectra of 254 and 366 nm light irradiation. They could be thought as equivalent, considering the CLA was so highly sensitive that the spectra were often lacking reproducibility. Oxygen molecules absorbence on the surface of the sample, which provided a symmetric, strong and broad emission peak at 520 nm, affected the chemiluminescence's spectrum. An assistant line is inserted to indicate this oxygen peak at 520 nm at each spectrum in Fig. 7, facilitating to observe the other emission peaks. The emission peaks at 570 and 610 nm were strongly observed when irradiated by both wavelength light, comparing the background emission, 1000 count/s. These peaks were obtained reproducibly in triplicate measurements and did not declined even though the sample was heated to 70 °C.

Table 1
Rate of photo degradation for ketoprofen exposed by ultra violet for several minutes

Irradiation time (min)	Degradation (%)
0	0
5	1.8
10	2.1
30	48.1
60	90.9

% of degradation was calculated by the following equation: $(A_I - A_R)/A_I \times 100$ in which, A_I: HPLC peak area of ketoprofen at initial (0 min), A_R: HPLC peak area of ketoprofen at each irradiation time.

3.4. Determination of superoxide anion by the on-line photo irradiated superoxide anion detection system

The ketoprofen was injected into the system, carried with TNM to the photoreaction coil and irradiated by the chemical lamp. On the HPLC chromatogram the new peak was observed at 2.8 min besides ketoprofen and its three photo degradation products. This peak was not appeared without ketoprofen, TNM or the photo irradiation. TNM was reacted with superoxide anion, which was broken out from ketprofen by photo exposing, generating the extremely stable compound $(C{=}N{=}O_2)_3$ (Fig. 8). This system is so capable to control the exposing and the reaction time that the superoxide anions could be reproducibly detected. DMSO, determined as a superoxide anion generator by ESR spectrometry when exposed by light as well as ketoprofen, was also applied to the online system. It could be estimated that the amount of superoxide anion generated from exposed ketoprofen was as

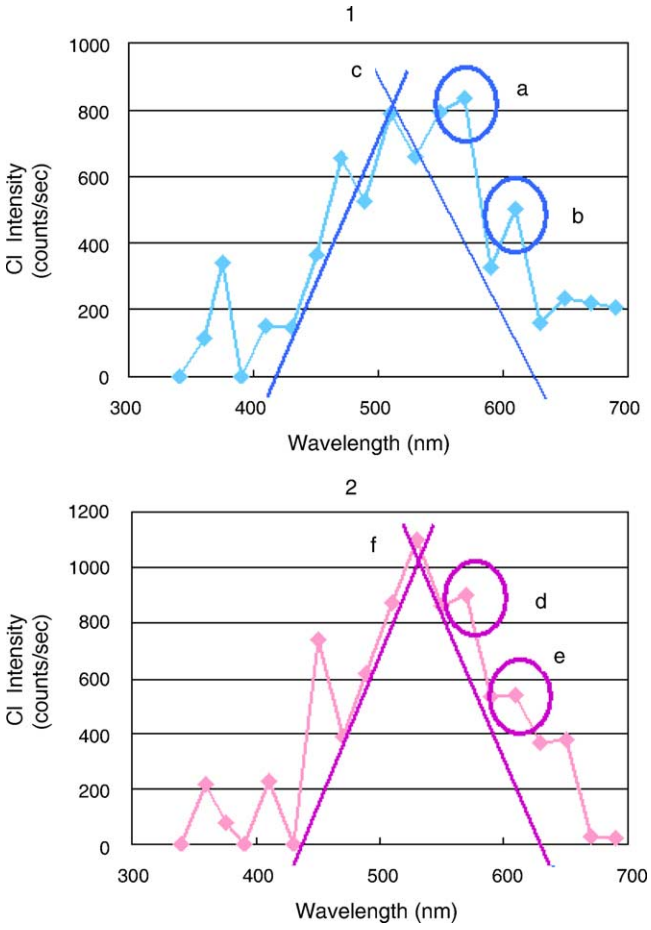

Fig. 7. CL spectra of photo-irradiation in air. (1) 254 nm, (2) 366 nm. The emission peaks a, d at 570 nm, and b, e at 610 nm. The assistant lines c and f indicate the peak from the oxygen molecules.

1.1 times as one delivered from exposed DMSO depend on the ratio of peak areas at retention time 2.8 min.

3.5. Speculation of photo degradation of ketoprofen

The three photo degradation products, isolated by TLC and HPLC, were analyzed using mass and NMR spectrometry. The products A and B were identified as 2,3-bis-(3-benzophenyl)butane and 3-acethylbenzophenone respectively by the mass fragmentation and NMR assignment. The product C was easily decomposed after isolation, so that the sufficient amount was not obtained to have a NMR spectrum. It was estimated the product C could have a chemical structure of Fig. 9b, which indicates the molecular weight 240.

3.6. Stability of free radicals

The symptoms of the photosensitization caused by ketoprofen have been appeared immediately or from several days after the exposure of light. We therefore traced the free radicals' behavior using ESR when intercepted from light. The ketoprofen solution was exposed by UV for 60 min, transferred into a ESR sample tube, obtained ESR spectra at every 90 s (Fig. 10). The exposed solution was otherwise shielded to intercept the light and applied to HPLC analysis to monitor ketoprofen and the photo degradation products at every 15 min. The ESR spectra indicated that the free radicals did not disappeared immediately in spite of interception from the UV light, but continued for 12 min while declining gradually. It was confirmed that the free radicals and the active oxygen species were still generated in exposed ketoprofen solution after intercepting the irradiation of the UV light. According to the HPLC analysis, the degradation products were increased gradually (Fig. 11). The products A and B were predominantly swelled, and the product C was slightly increased.

4. Discussion

The white fluorescence lamp, the chemical lamp and the ultra violet lamp radiates the light of the wavelength of over 350, 300–430 and around 254 nm, respectively. The fluorescence lamp and chemical lamp correspond to the room light and the sunbeam, respectively. The chemical lamp could be the most appropriate to estimate the influence of the sunbeam for ketoprofen. No clear signal was however obtained in the ESR spectrum of ketoprofen and DMPO solution when irradiated by chemical lamp. There were no significant differences of decomposition way among the three lamps, although the decomposition speeds were different (Fig. 3). In the CLA analyses, the similar emission spectra were obtained at 254 and 366 nm irradiation (Fig. 7). It was considered that the forms of free radicals and active oxygen species generated from ketoprofen could be equivalent between irradiation by the UV light of long wavelength and of short wavelength.

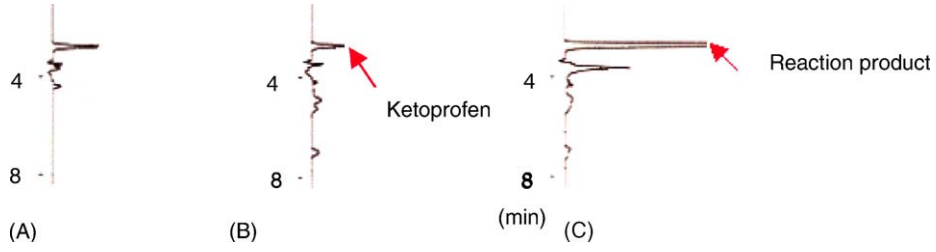

Fig. 8. Detection of superoxide anions by TNM method: (A) TNM, (B) ketoprofen, (C) TNM + ketoprofen.

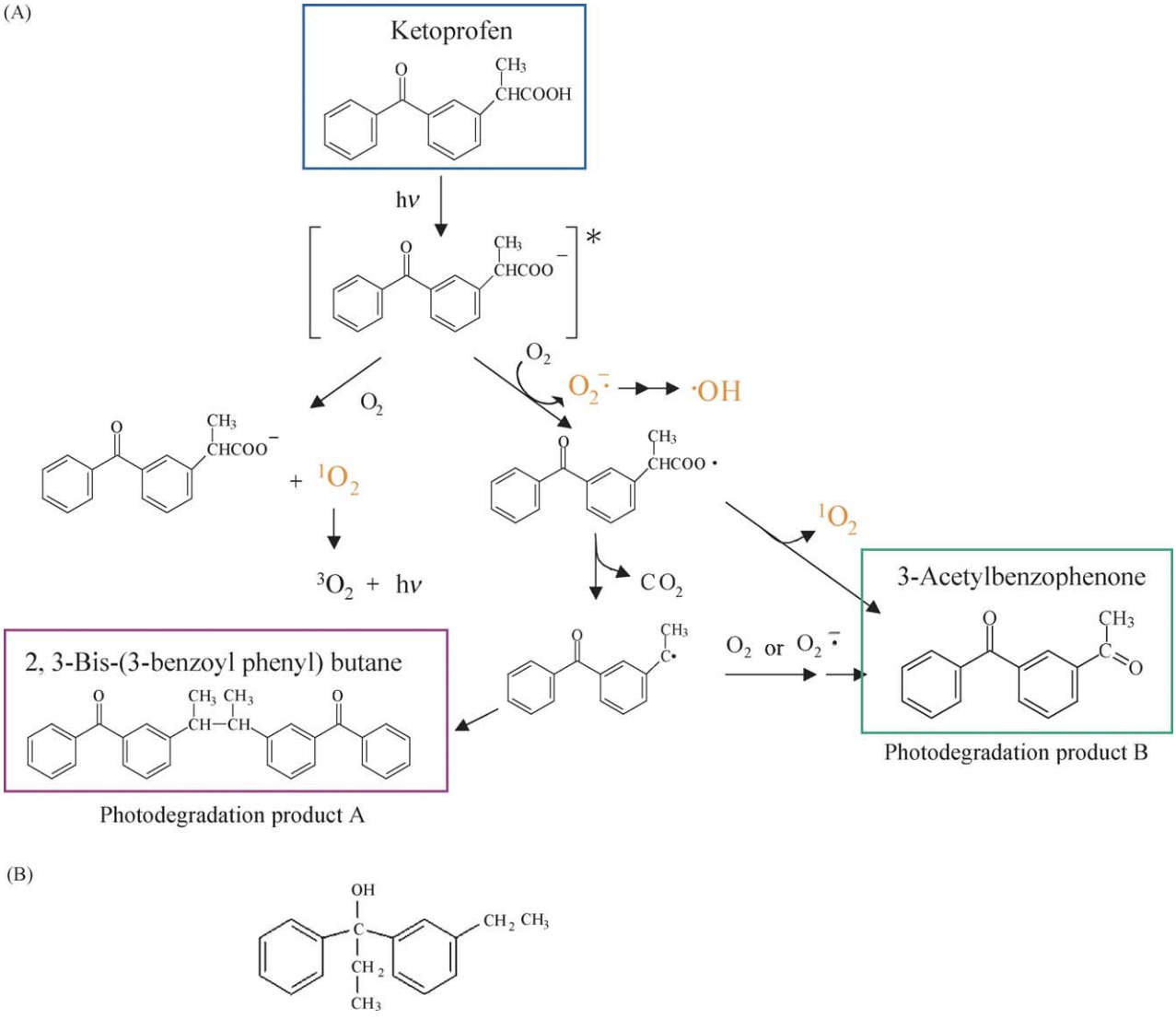

Fig. 9. (A) Estimated photodegradation pathway of ketoprofen; (B) estimated chemical structure of photodegradation product C.

At ESR experiments, no free radicals were observed in ketoprofen and DMSO solutions without DMPO, even though irradiated by the 254 nm UV lamp. When the trapping reagent (DMPO) was added to the sample solutions after irradiation, or the sole DMPO solution was irradiated, no signals were obtained in the ESR spectra. It was therefore considered that the generated free radicals from ketoprofen have extraordinary reactivity and their lifetime could be less than 10^{-6} s and DMPO could work to maintain the lifetime of free radicals.

Injecting the ketoprofen or DMSO to the on-line system, the peak of the reaction product of TNM and the superoxide anion was clearly detected (Fig. 8). This result could demonstrate that the superoxide anion was generated by the chemical lamp as well as the 254 nm UV lamp, and the superoxide anion detected at the ESR experiment was not generated from light exposed DMPO, but light exposed ketoprofen and DMSO. The two methods of the on-line photo-irradiated superoxide detection systems and the off-line photo-irradiation/ESR experiments were not specific for ketoprofen to determine photo induced superoxide anion, but they could be multiply used. The on-line photo-irradiated superoxide detection systems enabled to instantly and simply determine fresh superoxide anions just after generated from the photo-exposed chemical substances, which is generally known unstable.

In CLA spectra, although the predominant emission peak was specifically obtained at 1269 nm when the singlet oxygen was transferred to the ground state (the triplet oxygen) ($^1O_2 \rightarrow {}^3O_2 + h\nu$), this peak could not be detected because the detection range of the instrument used in this study was 330–700 nm. The singlet oxygen is however at the several energy levels, and the chemiluninescence are observed at 634, 478 and 381 nm [17]. The emission peaks at 520 nm could attribute to the oxygen molecules on the surface of ketoprofen crystal. The emission peaks at 570 and 610 nm were strongly and reproducibly observed when irradiated by 254

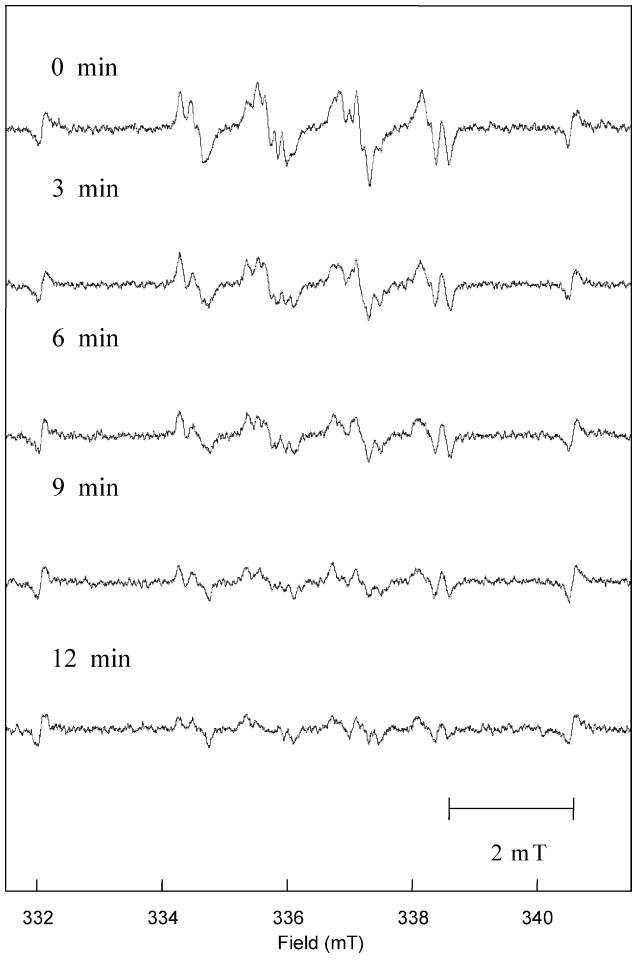

Fig. 10. ESR spectra of exposed ketoprofen solution containing DMPO, shielded from light.

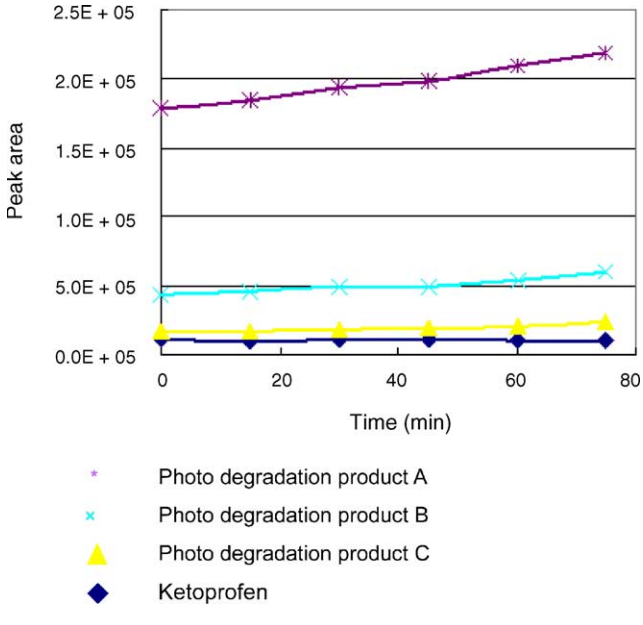

Fig. 11. HPLC analysis of ketoprofen and the photo degradation products in the exposed solution for 60min, plotting at every 15 min after intercepted from the light.

and 366 nm wavelength lights (Fig. 7). It was considered that the emission peak at 610 nm could attribute to the singlet oxygen, generally observed at 634 nm, and the emission peak at 570 nm could be observed when excited carbonyl group return to the ground state of carboxylic acid at the propionic acid group in ketoprofen. Free radicals and active oxygen species could be generated from ketoprofen molecules at not only the solution state but also at the solid condition when irradiated by the sunbeam.

According to the results of all of experiments, the pathway and mechanism of photodecomposition of ketoprofen could be suggested as Fig. 9a. It is considered that the excited carboxyl anion is primarily generated from ketoprofen by the UV light energy. It could be changed to the carboxylic anion radical, generating superoxide anion and hydroxyl radical. The carboxylic acid radical is turned to 3-(benzophenyl)ethane radical through de-carboxylic acid pathway, and dimerized to the product A. The product B could be produced from the carboxylic acid radical or 3-(benzophenyl) ethane radical, while generating superoxide anion or singlet oxgen. At ESR profile of ketoprofen (Fig. 5), the signals' intensities of the hydroxyl radical and superoxide anion were not significantly different among the spectra of 5, 10 and 30 min irradiation, although residual ratios of ketoprofen were notably different at Table 1. It is supposed that the excited carboxyl anion could be continuously developed and changed to the product A. Upending domination for the ratio of the product A over residual ketoprofen, generation of these radicals' could be diminished. The photo degradation product A could have no property to produce free radicals as well as ketoprofen. Once ketoprofen is sufficiently exposed by the ultraviolet light, the free radicals and the active oxygen species could be continuously generated after shield from the light, since the excited carboxyl acid anion of ketoprofen could be comparatively stable. It should be concerned that the risk of phototoxicity of ketoprofen could be increased with longer and stronger exposure by sunbeam despite dose and dosage form.

5. Conclusions

Ketoprofen is decomposed by the ultraviolet, generating the active oxygen species, the superoxide anion, the hydroxyl radical and the singlet oxygen. The amount of superoxide anion generated from exposed ketoprofen was as much as one from DMSO. The pathway of the photodecomposition and the mechanism of generation of the active oxygen species were estimated according to the results of identifiable analysis. The excited carboxyl anion of ketoprofen attributes to the generation of the active oxygen species, and changed to the photo degradation product A or B though de-carboxylic acid pathway. It was considered the excited carboxyl anion is comparatively stable, therefore the radicals were detected in ESR spectra after shielded from the light, although the lifetime of these radicals are extremely short. We would

like to apply these results of this study to the examination for preventing the phototoxicity and photosensitization of ketoprofen.

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