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Identification of 6-aminochrysene photoproducts and study of the effect of a humic acid and riboflavin on its photolysis

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Abstract

The effect of the photosensitizer riboflavin (0, 10, 25, 50, 100 μ M) and a river humic acid (0, 20, 40, 80 ppm) on the photolysis of 6-aminochrysene (6AC) in 10 mM PBS solution (pH 7.0) was studied. The presence of riboflavin significantly enhanced photolysis rate of 6AC. The photo-transformation half-life of 6AC was 1 min or 36 min respectively in the presence or absence of riboflavin. The humic acid inhibited the photo-transformation rate of 6AC. The photo-transformation half-lives of 6AC were 37, 56, 92 min at 20, 40, 80 ppm humic acid, respectively. By using LC-MS, the main 6AC photoproduct identified was 5,6-chrysene-quinone along with some minor products. Both 6AC and 5,6-chrysene-quinone exhibited photoinduced cytotoxicity. A photochemical transformation pathway for 6AC was derived.

Keywords

6-Aminochrysene; Photosensitizer; Photolysis; Cytotoxicity; Humic acid; Riboflavin

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of toxic environmental pollutants [1–4]. Many of them are known carcinogens and mutagens [1, 2]. Photolysis transforms many organic pollutants to less toxic or harmless products. However, in the presence of natural or simulated sunlight, many PAHs become more toxic to aquatic organisms at concentrations well below PAHs' aqueous solubility limits [4–6]. This may occur via a photosensitization reaction to produce reactive chemical species [7, 8] or via photo oxidation to produce more toxic diols or quinones [4, 9].

Chrysene is one of the basic PAHs. The photoproduct mixture of chrysene was complex and its speciation was not reported [10]. Chrysene and 6-aminochrysene (6AC) have been shown to cause UVA light-induced DNA cleavage and genotoxicity [11, 12]. The cytotoxicity and mutagenicity of other structurally related PAHs such as 6-nitrochrysene, 6-hydroxychrysene, 2- and 5-aminochrysene had also been reported [13–17]. In this study, liquid chromatography coupled with mass spectrometer detector (LC-MS) was used to separate and identify the photoproduct mixture of 6AC. The cytotoxicity of 6AC and its main photoproducts 5,6-chrysenequinone (5,6-CQ) was tested with spread plate method.

Photosensitizers have been used to facilitate photochemical transformation of selected organic compounds. The mechanism may involve redox reactions. Natural aquatic dissolved substances such as humic acids (HA) were found to be effective as photosensitizers for the

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transformation of PAHs [16, 17]. However, HA could become light filters by attenuating light radiation. Riboflavin, a secreted organic compound from phytoplankton in natural waters, has been reported to be an effective sensitizer to enhance photochemical transformation of many organic compounds in aqueous solutions [18–20].

Since riboflavin and river humic acid have significant influences on the environmental fate of many organic contaminants (including PAHs) in natural environment [16–20], we must consider their influences in order to predict the environmental fate and effect of PAHs. In this paper the stable photoproduct of 6AC (5,6-CQ) was identified and the photosensitizing effect of riboflavin and a river humic acid on the photochemical fate of 6AC in a PBS solution was studied.

2. Experimental

2.1. Materials

6AC and riboflavin (Aldrich Chemical Co., Milwaukee, Wisconsin) were used as received. Standard river humic acid was purchased from International Humic Substances Society. River humic acid solutions were prepared in different concentrations with 1 mM sodium phosphate buffer (PBS, pH 7.0).

2.2. Photo-transformation of 6AC

To 150-mL quartz flasks (Quartz Scientific, Fairport Harbor, Ohio), 24 mL of media solution (river water, pure water with or without a certain concentration of humic acid or riboflavin) and 1 mL 250 μ M 6AC in DMSO were added and shaken to mix. The flasks were exposed to a 100 W UVA lamp (type B, UVP Inc., Upland, CA) indoors for different time periods. The UVA intensity is 8.9 mW/cm² at the irradiation distance of 15 cm.

Transformation (disappearance of 6AC) rate constants were calculated by assuming the reactions were first-order. The first-order rate expression is given by $\ln(C_0/C) = k_p t$, where C_0 and C refer to the concentrations of 6AC at t_0 and t , respectively, and k_p is the first-order photolysis rate constant in min⁻¹. The half-life of 6AC was determined by $t_{1/2} = 0.693/k_p$ [21].

The HPLC system is a Waters Millennium system with two Waters 515 pumps, a Waters 717 plus Autosampler and a Waters 996 photodiode array (PDA) detector. Separation was carried out on a Lichrospher® 100 RP-8 column (25 cm $\times$ 4.0 mm I.D., 5 μ m) (Hewlett Packard, USA) at a flow rate of 1.0 mL/min with gradient elution of acetonitrile/water mixture (60/40 to 90/10 v/v from 0 to 7 min, held at 90/10 v/v from 7 to 10 min, then 90/10 to 60/40 v/v from 10 to 12 min). The wavelength of PDA was set at 273 nm and the temperature of the autosampler was controlled at 12°C.

2.3. 6AC photoproduct identification by LC-MS

After photolysis, the solution was concentrated with a rotary evaporator (Büchi Waterbath B-480 and Büchi Rotavapor R-114) at 38°C. The extracted solution was subjected to LC - MS analysis.

The LC-MS system is a Finnigan LCQ^{DUO} MS system with SpectraSYSTEM P4000, AS3000, UV6000LP. Separation was carried out on a Lichrospher® 100 RP-8 column (25cm $\times$ 4.0mm I.D., 5 μ m) (Hewlett Packard, USA) at a flow rate of 1.0 mL/min with gradient elution of acetonitrile/water mixture (40/60 to 90/10 v/v from 0 to 10 min, held at 90/10 v/v from 10 to 18 min, then 90/10 to 40/60 v/v from 18 to 20 min). The wavelength of PDA was set at 254 nm and the temperature of the autosampler was controlled at 12°C. The mass spectra were acquired at 1 scan/s over a m/z scan range of 50–500. The MS-MS mode

was used. The capillary and APCI vaporizer temperature were 180 and 450°C, respectively. Nitrogen was used as both sheath and auxiliary gas. The mass spectrometer was operated in the positive or negative ion detection mode as needed.

2.4. 6AC and 5,6-CQ cytotoxicity determination by the spread plate method

The photolysis process was carried out in 150-mL Erlenmeyer quartz flasks without shaking (Quartz Scientific, Fairport Harbor, Ohio). 6-AC or 5,6-CQ was added to reach a final concentration of 10 µM with 50 mL fresh river water. Afterwards they were incubated in a running water bath under natural sunlight outdoors at noontime for 36 min.

Serial dilutions were made for the water samples. To make the dilutions, 9 mL of distilled, autoclaved water was placed in a sterile glass, screw-cap test tubes, after which 1 mL of the sample water was placed in the tube. The solution was mixed using a vortex mixer (VWR Scientific) for approximately 30 seconds. Aseptic technique was used while 0.1 mL of the solution was pipetted onto a clean nutrient agar plate and spread with an ethanol-sterilized glass spreader. The plate was then inverted and placed in an incubator at 24 °C. The results were counted in 2 days. The following formula was used to calculate the number of colony forming units (cfu) per mL:

$$\text{Number of colonies} \times \text{dilution factor} \times 10 = \# \text{CFU/mL}$$

2.5. Statistical analysis

Statistical analysis of results was conducted by factorial arrangement of treatments in a complete randomized design using General Linear Model by SAS [22]. LSMEANS were used to separate means or combination of means [23].

3. Results and Discussion

3.1. Separation and identification of 6AC photoproducts

Fig. 1a. shows the HPLC chromatograms of 6-aminochrysene (6AC) and its photoproducts derived from the solution containing 20 ppm riboflavin under UVA light irradiation. Peak 1 = 6AC, peak 2 = 5, 6-CQ, peak 3 = riboflavin, and other small unidentified peaks. The main photoproduct peak in this figure is peak 2, identified as 5, 6-CQ. The positive identification of this product was accomplished by co-eluting with an authentic sample and comparison of their UV-Vis absorption spectra. Fig. 1b shows comparison of the HPLC chromatograms of 6AC and its photoproducts (bottom), 5,6-CQ authentic standard (middle) and 6AC standard (upper). 5,6-CQ, a photoproduct of 6AC, has the same retention time as the authentic 5,6-CQ standard. After comparison, UV-Vis absorption spectra of 5,6-CQ standard (a) were found to be identical to that of 6AC photoproduct 5,6-CQ (b) (Fig. 2). These evidences strongly indicate that the main photochemical product of 6AC is 5,6-CQ.

Identification of 6AC photoproducts was further conducted by LC/MS/MS. Fig. 2. also shows the mass spectra of 6AC and 5,6-CQ. The mass spectrum with a molecular peak at 243.3 is for 6AC (MW=243) (Fig. 2c) and the other mass spectra with a molecular peak at 258.1 is for 5,6-CQ (MW=258) (Fig. 2d). By using MS/MS selective ion scan technique, the following possible minor photoproducts were also found based on their masses, 6-nitrosochrysene (6-NOC, MW=257), 6-nitrochrysene (6-NO₂C, MW=273), 6-hydroxyaminochrysene (6-HONC, MW=258), and dimers (MW = 484, 498, and 514).

To propose a suitable mechanism for 6AC photo-transformation, experiments were carried out using two singlet oxygen scavengers (NaN₃ and L-histidine) and a free radical scavenger

($\text{Na}_2\text{S}_2\text{O}_3$). Without a quencher, 6AC was photo-transformed 44.5% and 49.5% after 15 and 30 min respectively; with NaN_3 , the corresponding numbers were 19.8% and 48.1%; with L-histidine, the numbers were 9.2% and 46.6%. These data suggest singlet oxygen involves in the 6AC photo-transformation. With $\text{Na}_2\text{S}_2\text{O}_3$, there was no observable 6AC photo-transformation after 15 and 30 min of irradiation. This result strongly indicates that reactive oxygen species play an important role in the 6AC photo-transformation.

Based on the photoproducts identified, Scheme I is proposed as the photo-transformation pathway of 6AC. Nitro-reduction has been reported as the metabolic pathway that leads to mutagenesis of nitro-PAHs [11, 24]. Furthermore, enzyme reduction of nitro-PAHs produces nitroso, hydroxyamino-, and amino- compounds and leads to DNA adducts formation [25]. Our finding of 6AC photoproducts includes 6-nitrosochrysene, 6-nitrochrysene, 6-hydroxyaminochrysene can help to explain the photo-induced toxicity of 6AC.

3.2. Cytotoxicity of 6AC, 5,6-CQ and its photoproducts

After the microbial assemblages in fresh river water were exposed to 10 μM 6AC under outdoor sunlight irradiation for 36 min, viability counts of heterotrophic bacteria assemblages were inhibited by 82% relative to the light control group. However, no effect on viability count was observed after exposure to 6AC in the dark (Fig. 3). After exposure to 10 μM 5,6-CQ under outdoor sunlight irradiation for 36 min, viability counts of heterotrophic bacterial assemblages were inhibited by 68% relative to the light control group. However, no effect on viability count was observed after exposure to 5,6-CQ in the dark (Fig. 3). These data were also analyzed with a two factors (chemical and light) experiment program in SAS. It was found that both chemical and light have significant effect in causing cytotoxicity (both $p < 0.05$). There was no significant difference between 6AC and 5,6-CQ in causing photo-induced cytotoxicity. 5,6-CQ is the main photoproduct of 6AC and it is not cytotoxic in dark.

3.3. Effect of a river humic acid and riboflavin on 6AC photolysis

The effect of a river humic acid on 6AC photo-transformation is shown in Table 1. 10 μM 6AC in PBS solution (1 mM, pH 7.0 containing river humic acid) was exposed to UVA irradiation for 5, 10, 20, 40, 90, and 120 min. The half lives ($t_{1/2}$) of 6AC, determined by the method described in the experimental section 2.2, were 36.1, 37.1, 56.3, 92.4 min at river humic acid concentrations of 0, 20, 40, 80 ppm, respectively. At concentrations < 20 ppm, there was no obvious effect. With increasing concentrations, the river humic acid inhibits the photo-transformation of 6AC. This is possibly due to the fact that the river humic acid solution is colored and absorbs light to act as a light filter [16].

In our previous study, we also found that the photolysis rate of 1-aminopyrene (1-AP) and 1-hydroxypyrene (1-HP) varies according to the type of humic substances [16,17]. The half lives of 1-AP were 17.4, 6.7, 6.5, 6.9 min at river humic acid concentration 0, 20, 40, 80 ppm respectively. The half lives of 1-HP were 18.0, 15.1, 18.4, 22.1 min at river humic acid concentrations 0, 10, 20, 40 ppm respectively. With addition of the river humic acid at test concentrations, the photolysis rate of 1-AP was enhanced by about 3 fold. As for 1-HP, no significant influence was observed. However at the equivalent river humic acid concentrations, the photolysis rate of 6AC was inhibited by about 3 fold. Therefore, the influence of the river humic acid on the photolysis rate of substituted PAHs differs, depending on the type of the substituent.

The effect of riboflavin on 6AC photo-transformation is shown in Table 1. 10 μM 6AC in PBS solution (1mM, pH 7.0) containing riboflavin was exposed to UVA light irradiation for

0.5, 1.0, 1.5, 2.0 and 3.0 min. The half lives determined are 36.1, 1.1, 1.0, 1.3, 0.9 min at riboflavin concentrations of 0, 10, 25, 50, 100 μM , respectively. Within the concentration range of 10–100 μM , no significant difference in riboflavin-enhanced 6AC photolysis rate was observed. Relative to the control, riboflavin shortens the half life of 6AC by about 36 fold. Therefore, as a photosensitizer for 6AC, riboflavin is effective at a concentration of as low as 10 μM . The mechanism of the photolysis rate enhancement may involve redox reactions via type I photosensitization [20, 26].

In summary, 5,6-CQ was found to be the main photoproduct of 6AC. Both 6AC and 5,6-CQ exhibited photo-induced cytotoxicity. However, 5,6-CQ itself showed no cytotoxicity without light. Other photoproducts such as 6-NOC, 6-NO₂C, and 6-HONC might also contribute to the phototoxicity of 6AC. Addition of riboflavin enhanced the photolysis rate of 6AC by 36 times while addition of the HA caused photolysis inhibition by up to 3 times.

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3. Abbreviations

PAHs	Polycyclic aromatic hydrocarbons
6AC	6-aminochrysene
5,6-CQ	5,6-chrysene quinone
6-NOC	6-nitroso-chrysene
6-NO₂C	6-nitro-chrysene
6-HONC	6-hydroxyaminochrysene
HA	Humic acid
PBS	Phosphate buffer solution
1-AP	1-aminopyrene
1-HP	1-hydroxypyrene
SAS	A statistical analysis program
cfu	Colony forming units

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Fig. 1.

Fig. 1a. HPLC chromatograms of 6-amino chrysene (6AC) and its photoproducts in 20 ppm riboflavin solution under UVA irradiation. Peak 1 = 6AC, peak 2 = 5, 6-CQ, peak 3 = riboflavin. Exposure time: 0.5, 1, 1.5, 2, 2.5 min (bottom up). Fig. 1b. Comparison of HPLC chromatograms of 6-aminochrysene (6AC) and its photoproducts with that of 5,6-CQ standard.

Fig. 2. UV-Vis absorption spectra of 5,6-CQ standard (a) with that of 6AC photoproduct 5,6-CQ (b). Mass spectra of 6AC (c) and its photoproduct 5,6-CQ (d). The mass spectrometer detector: APCI vaporizer, 1 scan/s, m/z scan range 50–600.

Fig. 3.

Comparison of viable counts of bacterial assemblages in different exposure groups.

Treatment-darkness: 1= control, 2 = 6AC (10 μ M), 3 = 5,6-chrysne-quinone (10 μ M). Light exposure: 4= control, 5 = 6AC (10 μ M), 6 = 5,6-chrysne-quinone (10 μ M).

Scheme I.

Proposed photo-induced transformation pathways of 6-aminochrysene

Table 1

6-Aminochrysene photolysis half life ($t_{1/2}$) in PBS buffer solution containing either a river humic acid or riboflavin

River Humic Acid, ppm	$t_{1/2}$, min	Riboflavin, μ M	$t_{1/2}$, min
0	36.1 $\pm$ 1.5	0	36.1 $\pm$ 1.5
20	37.1 $\pm$ 2.5	10	1.1 $\pm$ 0.4
40	56.3 $\pm$ 3.7	25	1.0 $\pm$ 0.5
80	92.4 $\pm$ 4.5	50	1.3 $\pm$ 0.4
		100	0.9 $\pm$ 0.4