Contribution of Glucuronic Acid and Sulfonic Acid Moieties during Photocatalytic Degradation of Estrogen Conjugates

Tatsuhito Mizuguchi, Yujin Shibayama, Kuniko Mitamura, and Kazutake Shimada*

Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan

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Contribution of glucuronic acid and sulfonic acid moieties during the photocatalytic degradation of estrogen conjugates, one of the endocrine disrupting chemicals, has been investigated. Estrogens were subjected to photocatalytic degradation using titanium dioxide immobilized on glass beads as a catalyst, whose time courses were measured by HPLC or liquid chromatography (LC)/MS/(MS). Estradiol and estrone, which have an unconjugated phenolic hydroxy group at the C-3 position, were gradually degraded by UV irradiation and nearly disappeared within 6 hr. 3-Desoxyestradiol, which does not have a phenolic hydroxy group at C-3 position, was also degraded like estradiol. The corresponding 17- or 3-glucuronide was degraded faster than the respective genin, estradiol or estrone. The double conjugate, estriol 3-sulfate 16-glucuronide, was also easily degraded within 3 hr. On the other hand, the degradation of estrogen 3-sulfate did not start within 2.5 hr but the reaction was completed within 6 hr. These data showed that the glucuronic acid moiety on the estrogen skeleton and sulfonic acid moiety at phenolic hydroxy group play an important role for this degradation reaction.

Key words —— estrogen, glucuronide, sulfate, photocatalytic degradation, HPLC, liquid chromatography/MS/(MS)

INTRODUCTION

In recent years, there has been increasing concern over the endocrine disrupting effect of the endogenous estrogens and xenoestrogens in environmental water through the excretions of humans, and domestic and farm animals.[1,2] It was reported that estradiol (E_2), which has the highest estrogenic potential, was photodegraded and mineralized to carbon dioxide in an aqueous solution using titanium dioxide (TiO_2) particles or films under UV light irradiation.[3,4] The photocatalytic degradation using a semiconductor photocatalyst as typified by TiO_2 is one of the safe, effective and promising environmental cleanup technologies. The reported mechanism of the photocatalytic degradation of E_2 suggested that the phenolic hydroxy moiety of E_2 was initially attacked by the hydroxyl radical, which was generated by the UV irradiation of TiO_2 in water.[3,5,6] In the preliminary paper,[7] we reported the time courses of the photocatalytic degradation of the estrogen conjugates using TiO_2 immobilized on glass beads as a catalyst, which were compared with those of the unconjugated estrogens. The obtained results showed that 3-glucuronide, in which phenolic hydroxy group was conjugated, was degraded much faster than the respective sulfate.

In this study we clarified the contribution of glucuronic acid and sulfonic acid moieties of estrogen conjugates during this photocatalytic degradation reaction (Fig. 1), which was done using HPLC or liquid chromatography (LC)/MS/(MS).

MATERIALS AND METHODS

Materials and Reagents —— E_2, estrone (E_1) and estriol (E_3) were donated by Teikoku Hormone Mfg. (Kawasaki, Japan). E_2 17-glucuronide ($E_2$17G), E_2 3-glucuronide ($E_2$3G), E_1 glucuronide (E_1G) and 3-methoxy E_1 (3-MeOE_1) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ethynylestradiol

*To whom correspondence should be addressed: Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920–1192, Japan. Tel. & Fax: +81-76-234-4459; E-mail: shimada@p.kanazawa-u.ac.jp

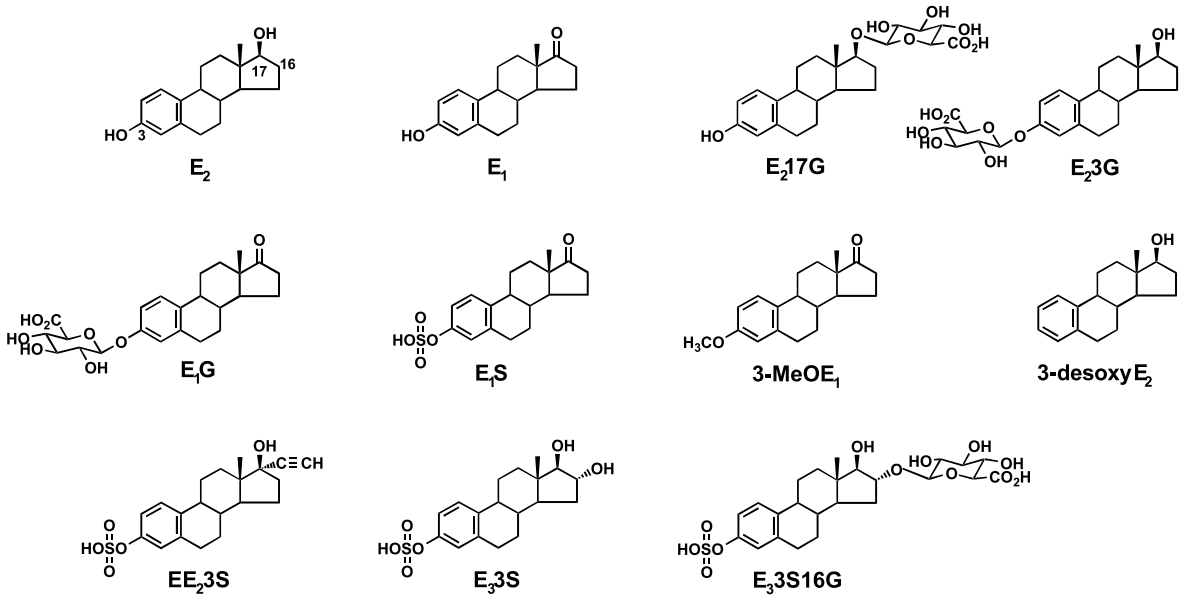

Fig. 1. Structures of Estrogens

Table 1. Analytical Conditions of Estrogens

Estrogen	UV (nm)	Mobile phase	Column	t_R (min)
E_2	280	MeOH-H_2O (7 : 3)	1	4.9
E_1	280	MeOH-H_2O (7 : 3)	1	4.7
$E_2$17G	280	MeOH-H_2O (1 : 1) containing 5 mM HCO_2NH_4	1	3.6
$E_2$3G	275	MeOH-H_2O (2 : 3) containing 5 mM HCO_2NH_4	1	4.5
E_1G	280	MeOH-H_2O (1 : 1) containing 5 mM HCO_2NH_4	2	5.5
E_1S	269	MeOH-H_2O (1 : 1) containing 5 mM HCO_2NH_4	1	6.0
3-MeOE_1	280	MeOH-H_2O (9 : 1)	1	5.4
3-desoxyE_2	(265)	MeOH-H_2O (9 : 1)	1	4.8
$EE_2$3S	280	MeOH-H_2O (3 : 2) containing 5 mM HCO_2NH_4	2	4.2
$E_3$3S	269	MeOH-H_2O (2 : 3) containing 5 mM HCO_2NH_4	2	4.9
$E_3$3S16G	(269)	MeOH-H_2O (3 : 7) containing 10 mM HCO_2NH_4	3	6.5

1. J'sphere ODS-H80 (150 × 4.6 mm i.d., YMC, Kyoto); 2. YMC-Pack Pro C18 (150 × 4.6 mm i.d., YMC); 3. Chemcobond ODS-W (150 × 2.1 mm i.d., Chemco, Osaka). Flow rate, 1 ml/min (except for $E_3$3S16G; 0.15 ml/min); column temperature, 40°C.

3-sulfate ($EE_2$3S) and $E_3$3S were purchased from Steraloids (New Port, RI, U.S.A.). E_1 sulfate (E_1S) was prepared in our laboratory from E_1 by the usual procedure using the chlorosulfonic acid-pyridine complex. E_3 3-sulfate 16-glucuronide ($E_3$3S16G)[8] and 3-desoxyE_2[9] were also prepared from E_3 and E_1 in our laboratory by known methods, respectively. The photocatalyst, BL2.5DX (diameter, 2.5 mm; membrane thickness, 1.0 μm: TiO_2 immobilized on glass beads: Lot No. 34040526), was purchased from Photo-Catalytic Materials (Komaki, Japan).

Oasis hydrophilic lipophilic balance (HLB) cartridges (60 mg, 3 ml) (Waters, Milford, MA, U.S.A.), Isolute C18 (EC: end-capped) cartridges (200 mg, 3 ml) (IST, Mid Glamorgan, U.K.) and Strata-X cartridges (60 mg, 3 ml) (Shimadzu, Kyoto, Japan) were successively conditioned with methanol (2 ml) and water (2 ml) prior to their use.

Apparatus —— HPLC was performed using a PU-980 pump (JASCO, Tokyo, Japan) equipped with a specific column and a SPD-10A UV detector (Shimadzu) (Table 1). A VL-4LC black light lamp (365 nm, 4 W) (Vilber Lourmat, Cedex, France) was used as the light source. The light power was measured by a UV LIGHT METER (290–390 nm; Lutron Asuka, Tokyo, Japan). LC/MS/(MS) was

Table 2. Pretreatment of Estrogens

Estrogen (Recovery rate; %, mean, $n = 2$)	Cartridge	Eluent
E_2 (87.3), $E_2$17G (93.1), 3-desoxyE_2 (62.0), $E_3$3S16G (63.9)	Oasis HLB	MeOH, 2 ml
E_1 (100.0), E_1S (100.0), E_1G (77.0), $E_2$3G (100.0), $EE_2$3S (82.7)	Isolute C18 (EC)	MeOH, 2.5 ml
3-MeOE_1 (91.5)	Isolute C18 (EC)	AcOEt, 2 ml
$E_3$3S (100.0)	Strata-X	MeOH, 2 ml

measured by a ThermoQuest LCQ (San Jose, CA, U.S.A.) under the following conditions together with those reported in Table 1: $E_3$3S16G: electrospray ionization (ESI) with negative ion and selected reaction monitoring (SRM) modes; capillary temperature, 175°C; capillary voltage, –10 V; tube lens offset, –30 V; ion spray voltage, 5 kV; sheath gas flow rate, 80 units; auxiliary gas flow rate, 20 units; relative collision energy, 20% (He); precursor ion *m*/*z* 543 $[M-H]^-$; product ion, *m*/*z* 367 [M-H-glucuronic acid]$^-$. 3-desoxyE_2: atmospheric pressure chemical ionization (APCI) with positive ion mode; vaporizer temperature, 550°C; capillary temperature, 180°C; capillary voltage, +10 V; tube lens offset, +10 V; sheath gas flow rate, 80 units; source current, 5 μA; selected ion monitoring (SIM) mode, *m*/*z* 239 $[M+H-H_2O]^+$.

Photocatalytic Degradation of Estrogens —— The previous method was slightly modified.[7] The ethanol solution of estrogen was diluted with water to 1 μM (the ethanol concentration was less than 0.3%, v/v). The photocatalytic glass beads (*ca*. 13.6 g) were spread so as to cover the bottom of a glass petri dish (diameter, 8 cm), and the estrogen solution (1 μM, 15 ml) was placed in the dish. The petri dish was placed in a light shielded box [22.5 (wide) × 13.5 (depth) × 8 cm (height)], and irradiated by a black light lamp at a distance of 8 cm for 6 hr. Five hundred microliters of solution was sampled every 30 min and then subjected to a specific cartridge as shown in Table 2. After washing with water (1.5–2 ml), the estrogen was eluted with an organic solvent and evaporated under a N_2 gas stream. The residue was dissolved in methanol (50 μl) and an aliquot was used for the HPLC or LC/MS/(MS).

The estrogen solutions (1 μM, 500 μl) of the above reaction mixture (without UV irradiation) were subjected to the pretreatment procedure and then analyzed by HPLC or LC/MS/(MS) as described above which were used as the 0 min illuminated samples. The peak areas of the estrogens at 0 min were taken as 1 and the relative values obtained from the reaction mixtures were measured. The absolute recovery rates of more than 62.0% (mean, $n = 2$) were obtained in these pretreatments (Table 2).

RESULTS AND DISCUSSION

We first repeated the previous experiment[7] several times in order to clarify the contribution of glucuronic acid and sulfonic acid moieties of estrogen conjugates during this photocatalytic degradation. Although $E_2$3G, $E_2$17G, E_1G and E_1S showed the same degradation pattern as reported, E_2 and E_1 were not degraded under the previous conditions. Reasons, such as the lot number of the used photocatalyst, the used light power or existence of the other catalyst in the reaction mixture,[10] were considered, but no plausible explanation for this discrepancy was obtained, therefore, further experiments were necessary to clarify the phenomenon. These data prompted us to re-investigate the previous experiment, that is, the distance between the light source and the petri dish was shortened to about the half the previous value (from 16 to 8 cm) while the light power remained constant (*ca*. 600 μW/cm^2) by measuring it during each experiment. The time courses of the photocatalytic degradations of the estrogens using the photocatalytic glass beads were monitored by measuring the remaining substrate by UV-HPLC. An aliquot of the reaction mixture was subjected to a solid-phase extraction, concentrated and then analyzed by HPLC, because the UV detector did not have sufficient sensitivity to detect estrogens without pretreatment (Table 2). The double conjugate, $E_3$3S16G, which is one of the main metabolites of pregnant women,[11] and 3-desoxyE_2 could not be monitored by UV-HPLC due to interfering peaks coming from the used cartridge. LC/ESI-MS/MS in the SRM mode and LC/APCI-MS in the SIM mode

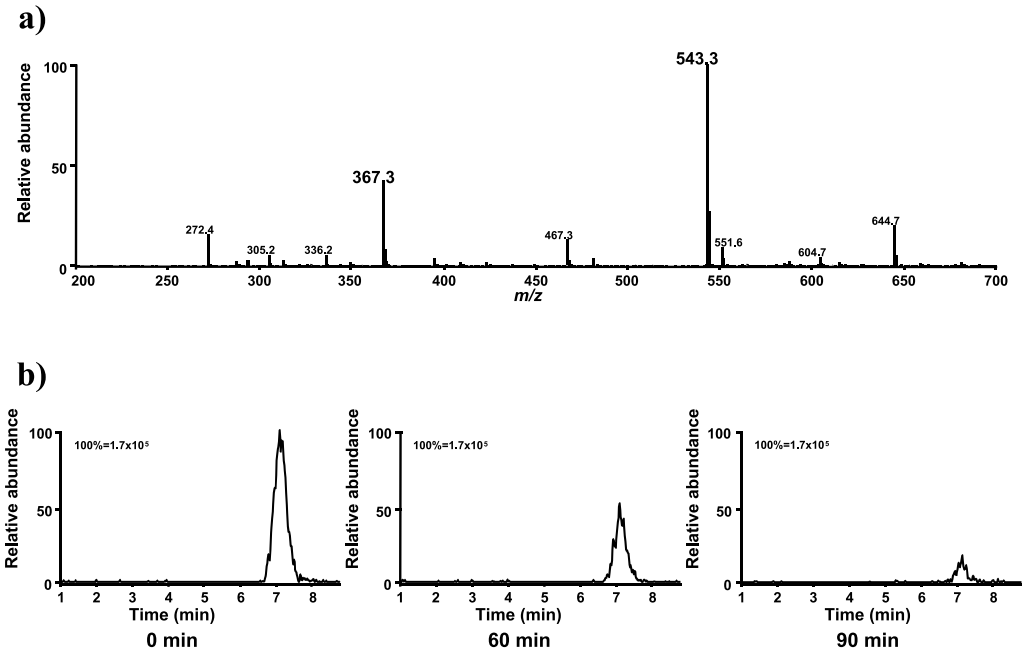

Fig. 2. LC/MS/MS Data of $E_3$3S16G

a) LC/MS/MS spectrum of $E_3$3S16G. Precursor ion, *m/z* 543 $[M-H]^-$; product ion, *m/z* 367 [M-H-glucuronic acid]$^-$. b) Product ion mass chromatograms of $E_3$3S16G after photocatalytic degradation.

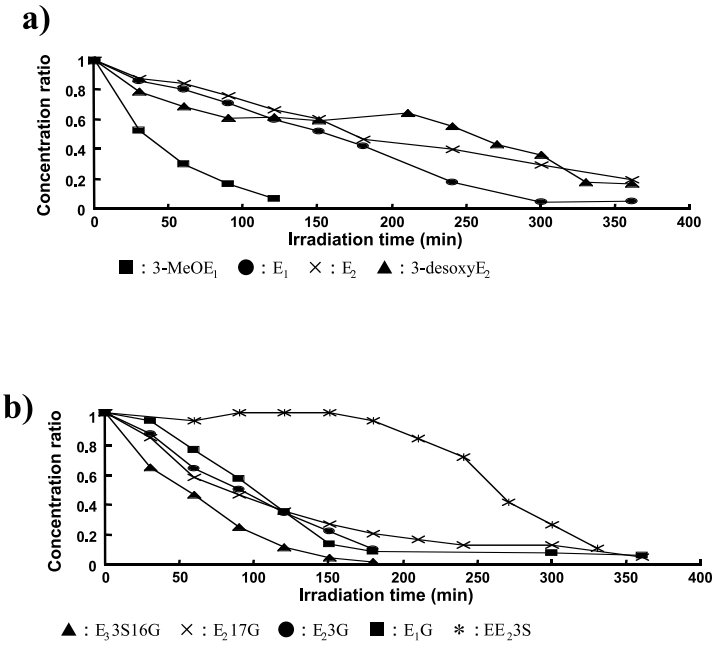

Fig. 3. Photocatalytic Degradation of Estrogens Using Photocatalyst (mean, $n = 2$)

a) Degradation of unconjugated estrogens. b) Degradation of conjugated estrogens.

were used for $E_3$3S16G (Fig. 2a) and 3-desoxyE_2, respectively. The separation conditions using HPLC or LC/MS/(MS) are summarized in Table 1.

The results of the photocatalytic degradation are shown in Fig. 3a, 3b and Table 3 (mean, $n = 2$). The degradation of E_2, which has an unconjugated phenolic hydroxy group at the C-3 position, was initiated by UV irradiation and nearly done within 6 hr. E_1 also showed the same degradation pattern. 3-DesoxyE_2, which does not have a phenolic hydroxy

Table 3. Degradation Rate Constants of Estrogens

Estrogen	Degradation rate constant (min^{-1}) by first order kinetics	r^2
E_2	0.0042	0.9691
E_1	0.0075	0.9050
$E_2$17G	0.0081	0.9719
$E_2$3G	0.0106	0.9190
E_1G	0.0092	0.8208
3-MeOE_1	0.0210	0.9900
$E_3$3S16G	0.0201	0.9526

r^2: coefficient of determination (> 0.8).

group at C-3 position, was degraded like E_2, but 3-MeOE_1 was degraded faster than E_1 (Fig. 3a). $E_2$17G, $E_2$3G, E_1G and $E_3$3S16G were almost completely degraded after 4 hr of UV irradiation like 3-MeOE_1 (Figs. 2b, 3b, and Table 3). On the other hand, the degradation of $EE_2$3S did not start even after 2.5 hr of UV irradiation, but the conjugate disappeared within 6 hr (Fig. 3b). The results of E_1S and $E_3$3S are not shown in Fig. 3b, but they showed the same pattern as that of $EE_2$3S. It is interesting that $E_3$3S16G was degraded faster than $E_3$3S, which suggests the contribution of the glucuronic acid moiety during this reaction. This phenomenon is compatible with the relationship between genin (E_2, E_1) and its monoglucuronide ($E_2$3G, $E_2$17G, E_1G); the latter was degraded faster than the former. The phenomenon was also confirmed on the reaction mixture containing both the genin and its monoglucuronide. Kitagawa *et al*. reported the photolytic degradation of saponin glucuronide to produce sapogenol, which was initiated by the excitation of the carboxy moiety of glucuronic acid followed by cleavage of the glucuronide bond.[12] The glycoside of the aromatic compound was also degraded to the respective genin by photolysis, and it is considered that the photoenergy was initially absorbed by the aromatic ring followed by the scission of glycoside linkage.[13] Although these photolytic degradations using high energy (such as a 500 W mercury lamp and vycor filter) in an organic solvent is quite different from the one using a photocatalyst (TiO_2) in water, some similarities have been observed regarding the contribution of the glucuronic acid moiety or aromatic ring during these degradations. Although the intermediate of the photocatalytic degradation of estrogen has been reported,[3] we could not find the intermediate, such as an unconjugated estrogen from estrogen conjugate, in the reaction mixture. E_2, 3-desoxyE_2 and $E_3$3S16G were almost quantitatively recovered from the reaction mixture under the stated control conditions, such as no UV light or catalyst. These data showed that the adsorption of estrogen on a photocatalyst was negligible and the photocatalyst effectively functioned.

All the above data suggested that the glucuronic acid moiety on the estrogen skeleton and sulfonic acid moiety at phenolic hydroxy group accelerates and disturbs this degradation reaction, respectively. But the further experiments are necessary to clarify this degradation mechanism. It is noted that endogenous estrogens (E_1, E_2, E_3) and synthetic ones (such as EE_2) are mainly excreted as conjugates such as glucuronide, sulfate or a double conjugate, and some are excreted as the methyl ether.[14] The obtained data are helpful for using this photolytic degradation technique to purify environmental water.

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