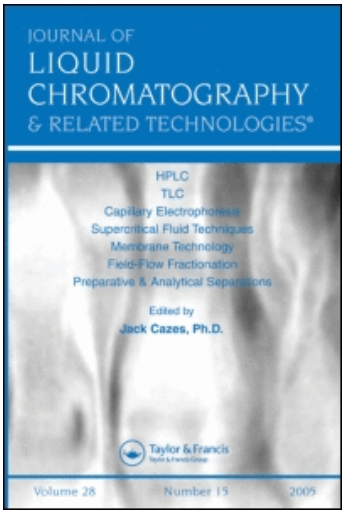


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Determination of the Effect of Hydrogen Peroxide on Photocatalytic Degradation of Estrogen Using HPLC

Tatsuhito Mizuguchi[a]; Satoko Sadaka[a]; Chie Ogasawara[a]; Kazutake Shimada[a]
[a] Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa, Japan

**To cite this Article** Mizuguchi, Tatsuhito , Sadaka, Satoko , Ogasawara, Chie and Shimada, Kazutake(2006) 'Determination of the Effect of Hydrogen Peroxide on Photocatalytic Degradation of Estrogen Using HPLC', Journal of Liquid Chromatography & Related Technologies, 29: 6, 903 — 911
**To link to this Article: DOI:** 10.1080/10826070500531458
**URL:** http://dx.doi.org/10.1080/10826070500531458

*Journal of Liquid Chromatography & Related Technologies*®, 29: 903–911, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500531458

# Determination of the Effect of Hydrogen Peroxide on Photocatalytic Degradation of Estrogen Using HPLC

**Tatsuhito Mizuguchi, Satoko Sadaka, Chie Ogasawara, and Kazutake Shimada**

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



**Abstract:** Estrogens were subjected to photocatalytic degradation using titanium dioxide immobilized on glass beads as a catalyst with or without the presence of hydrogen peroxide, whose time courses were measured using HPLC with a monolithic silica column. Hydrogen peroxide is effective for the photocatalytic degradation of estrone and estradiol, but not for the estrone- and ethynylestradiol-3-sulfates. The monolithic silica column is suitable for the fast analysis of the reaction mixture used for the photocatalytic degradation.

**Keywords:** Estrogen, Estrogen 3-sulfate, Photocatalytic degradation, Hydrogen peroxide, HPLC, Monolithic silica column

## INTRODUCTION

In a previous paper of this series, we clarified the contribution of the glucuronic acid and sulfonic acid moieties during the photocatalytic degradation of estrogen conjugates, one of the endocrine disrupting chemicals.[1] Estrogens were subjected to photocatalytic degradation using titanium dioxide immobilized on glass beads as a catalyst, whose time courses were measured by HPLC or LC/MS/(MS). Estrone ($E_1$) and estradiol

Address correspondence to Kazutake Shimada, Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. E-mail: shimada@p.kanazawa-u.ac.jp

($E_2$), which have an unconjugated phenolic hydroxy group at the C-3 position, were gradually degraded by UV irradiation and nearly disappeared within 6 hr. The corresponding 17- or 3-glucuronide was degraded faster than the respective genin, $E_1$ or $E_2$. 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 (3S) did not start within 2.5 hr, but the reaction was almost completed within 6 hr. The obtained data showed that the glucuronic acid moiety on the estrogen skeleton and sulfonic acid moiety at the phenolic hydroxy group play an important role in this degradation reaction. Tanaka *et al.* reported that the addition of hydrogen peroxide was effective for the photocatalytic degradation of organohalide compounds in a semiconductor suspension.[2]

In a previous paper, we clarified that a monolithic silica column provided satisfactory separation of the estrogens, with a lower retention factor ($k$) and lower column pressure than those obtained using the particle packed columns.[3]

These data prompted us to examine the effect of hydrogen peroxide on the photocatalytic degradation of estrogens, especially estrogen 3S, having a sulfonic moiety at the phenolic hydroxy group, and related steroids using HPLC with a monolithic silica column (Figure 1).



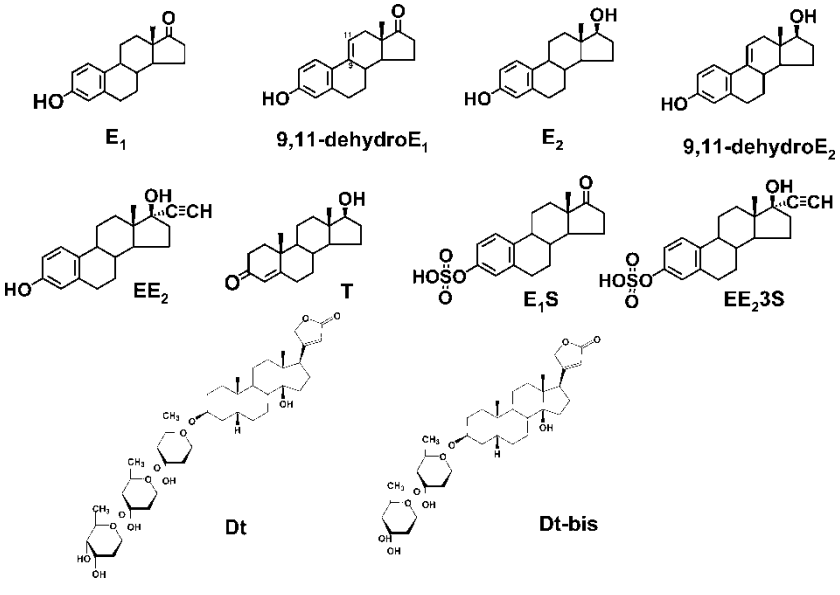


***Figure 1.*** Structures of steroids.

## EXPERIMENTAL

### Materials and Reagents

$E_1$ and $E_2$ were donated by Teikoku Hormone Mfg. (Kawasaki, Japan). $E_1S$ was prepared in our laboratory from $E_1$ by the usual procedure, using the chlorosulfonic acid—pyridine complex.[4] Ethynylestradiol ($EE_2$), testosterone (T), and digitoxin (Dt) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). $EE_2$3S was purchased from Steraloids (Newport, RI, USA). 9,11-Dehydroestrone (9,11-dehydro$E_1$) and 9,11-dehydroestradiol (9,11-dehydro$E_2$) were synthesized according to the reported method[5] and provided by Dr. Kohtani (Kanazawa University). Digitoxigenin bis-digitoxoside (Dt-bis) was prepared from Dt in our laboratory according to the reported method.[6] 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).

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. All the other reagents were of analytical grade and commercially available.



### Apparatus

The HPLC was performed using a LC-6A pump (Shimadzu) equipped with a monolithic silica column (Chromolith Performance RP-18e, 100 × 4.6 mm, Merck, Darmstadt, Germany) or a particle packed column (J'sphere ODS-L80, 5 μm, 150 × 4.6 mm, YMC, Kyoto) and a SPD-10A UV detector (Shimadzu) at the flow rate of 1 mL/min at 40°C. $NaNO_3$ was used for measurement of the dead time ($t_0$) at 220 nm. 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).

### Photocatalytic Degradation of Estrogens and Related Steroids

The photocatalytic degradation was performed as previously reported.[1] The ethanol solution of steroids 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 steroid solution (15 mL) was placed in the dish. In the case of the experiment using hydrogen peroxide, it was added at a final concentration of $1.32 \times 10^{-3}$ M.[2] 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 (*ca.* 600 μW/cm$^2$) at a distance of 8 cm for 6 hr. Five hundred microliters of solution was sampled every 30 min and then

subjected to a Strata-X cartridge, with or without the addition of the internal standard (IS) (Table 1). After washing with water (1.5–2 mL), the steroid 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 analysis (Table 2).

The steroid solutions (500 μL) of the above reaction mixture (without UV irradiation) were subjected to the pretreatment procedure and then analyzed by HPLC as described above, which were used as the 0 min illuminated samples. The peak areas of the steroids at 0 min were taken as 1 and the relative values obtained from the reaction mixtures were measured. Absolute recovery rates of greater than 83.6% (mean, n = 2) were obtained in these pretreatments (Table 1).

## Identification of Intermediate of Photocatalytic Degradation of Steroids

$E_1$ or $E_2$ (1 μM) was subjected to the photocatalytic degradation experiments for 3 hr and the entire reaction mixture was subjected to the Strata-X cartridge as described above, and then subjected to HPLC/UV for comparison with the authentic 9,11-dehydro$E_1$ and 9,11-dehydro$E_2$ using the monolithic silica column at 262 nm (Table 2). The obtained reaction mixture was also subjected to the following HPLC analysis using J'sphere ODS-H80 column (5 μm, 150 × 4.6 mm, YMC) at a flow rate of 1 mL/min at 40°C: 9,11-dehydro$E_1$ [MeCN-$H_2O$ (11 : 9), $t_R$ 14.3 min], 9,11-dehydro$E_2$ [MeOH-$H_2O$ (3 : 2), $t_R$ 7.4 min].

Dt (1 μM) was subjected to the photocatalytic degradation experiments for 6 hr and the entire reaction mixture was subjected to the Strata-X cartridge as described above, and then subjected to HPLC/UV for comparison with the authentic Dt-bis using the monolithic silica column at 230 nm (Table 2). The obtained reaction mixture was also subjected to the following LC/MS/MS analysis. An LC/MS/MS system, which consisted of an LC-10AT

***Table 1.*** Pretreatment of steroids

| Steroid | Eluent | Recovery rate; (%, mean, n = 2) | IS |
|---|---|---|---|
| $E_1$ | AcOEt, 1.5 mL | 88.6 | $E_2$ |
| $E_2$ | AcOEt, 1.5 mL | 86.9 | $E_1$ |
| $EE_2$ | AcOEt, 1.5 mL | 83.6 | $E_2$ |
| T | AcOEt, 1.5 mL | 83.6 | $E_2$ |
| $E_1S$ | MeOH, 2 mL | 89.9 | — |
| $EE_23S$ | MeOH, 2 mL | 92.3 | — |
| Dt | MeOH, 2 mL | 97.0 | — |

***Table 2.*** Separation of steroids using J'sphere ODS-L80 and Chromolith Performance RP-18e

| | J'sphere ODS-L80[a] | Chromolith performance RP-18e[b] | |
|---|---|---|---|
| Steroid | $k$ | $k$ | Mobile phase |
| $E_1$[c] | 5.3 | 2.5 | MeCN-$H_2O$ (2 : 3) |
| $E_1$[d] | — | 4.0 | MeOH-$H_2O$ (11 : 9) |
| 9,11-dehydro$E_1$[d] | 7.5 | 3.8 | MeOH-$H_2O$ (11 : 9) |
| $E_2$[c] | 7.4 | 3.5 | MeCN-$H_2O$ (2 : 3) |
| $E_2$[d] | — | 2.5 | MeOH-$H_2O$ (3 : 2) |
| 9,11-dehydro$E_2$[d] | 4.6 | 2.2 | MeOH-$H_2O$ (3 : 2) |
| $EE_2$[c] | 7.3 | 3.8 | MeCN-$H_2O$ (2 : 3) |
| T[e] | 5.5 | 3.2 | MeOH-$H_2O$ (3 : 2) |
| $E_1S$[e] | 7.9 | 3.1 | MeOH-0.1% $KH_2PO_4$ (5 : 6) |
| $EE_23S$[c] | 9.8 | 3.2 | MeOH-0.1% $KH_2PO_4$ (5 : 6) |
| Dt[f] | 10.9 | 5.3 | MeOH-$H_2O$ (7 : 4) |
| Dt-bis[f] | 10.7 | 4.7 | MeOH-$H_2O$ (3 : 2) |

$t_0$ (min), [a]1.7, [b]1.5; detection (UV, nm), [c]280, [d]262, [e]269, [f]230.



chromatograph (Shimadzu) coupled with an API 2000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA), was operated with electrospray ionization (ESI) in the positive-ion mode. A semi micro column, Develosil ODS-HG-5 (5 μm, 150 × 2.0 mm i. d.) (Nomura Chemical, Seto, Japan), was used at a flow rate of 0.2 mL/min at 40°C, and MeOH-5 mM $HCO_2NH_4$ (2 : 1, v/v) was used as the mobile phase (Dt-bis $t_R$ 6.9 min). The ionization conditions were as follows: ion spray voltage, 5 kV; heated nebulizer temperature, 500°C; ion source gas 1 (nebulizer gas), 70 psi, ion source gas 2 (turbo gas), 80 psi; declustering potential, 30 V; focusing potential, 350 V; entrance potential, 12 V; curtain gas, 20 psi. For the product ion scan [precursor ion, $m/z$ 652.5 $[M + NH_4]^+$; product ions, $m/z$ 635.3 $[M + H]^+$, 505.2, 487.2, 375.2 (100%)], $N_2$ was used as the collision gas (30 V).

## RESULTS AND DISCUSSION

We first compared the chromatographic behavior of the monolithic silica column (Chromolith Performance RP-18e) to that of the particle packed one. In a previous paper, we clarified that the J'sphere ODS-L80 gave the best results for the separation of estrogens among the examined particle packed columns.[3] These data prompted us to use the J'sphere ODS-L80

column as the particle packed one and the results are summarized in Table 2. The monolithic silica column eluted the steroid with a lower $k$ than those obtained with the particle packed one. The fast separation is suitable for not only the determination of the steroids, but also the environmental adaptation. These data prompted us to use the monolithic silica column for this experiment.

$E_1$, $E_2$, and $EE_2$ were subjected to the photocatalytic degradation experiments in the presence of hydrogen peroxide ($1.32 \times 10^{-3}$ M). These estrogens were degraded by first order kinetics without hydrogen peroxide, but in the presence of hydrogen peroxide, these were rapidly degraded with zero order kinetics as shown in Figure 2 and Table 3. But these estrogens were not degraded without the existence of the photocatalyst and UV irradiation. Kohtani et al.[7] and we suggested the existence of the intermediate (9,11-dehydro$E_2$) during the photocatalytic degradation of $E_2$. 9,11-Dehydro$E_1$ or 9,11-dehydro$E_2$ was found in the reaction mixture of the photocatalytic degradation of $E_1$ or $E_2$, respectively. These were confirmed by the HPLC chromatographic behavior using two different columns, which was compared with that of the authentic samples as shown in the experimental section. The degradation of $E_1$S and $EE_2$3S did not start before 2.5 hr, but the reaction was almost completed within 6 hr without the presence of

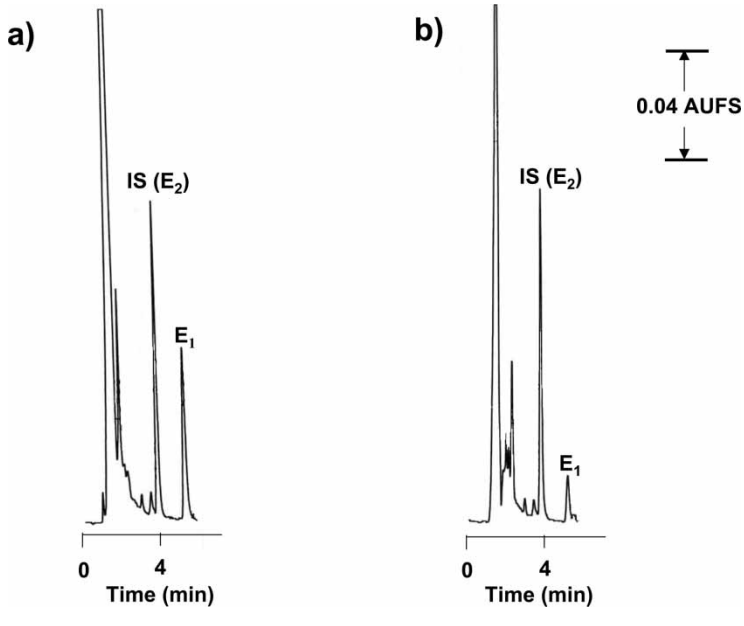


***Figure 2.*** HPLC chromatograms of photocatalytic degradation of $E_1$. Chromolith Performance RP-18e, MeCN-$H_2O$ (2 : 3), 1 mL/min, 280 nm; Irradiation time 60 min. a) without $H_2O_2$, b) with $H_2O_2$.

***Table 3.*** Degradation rate constants of steroids

| | Without $H_2O_2$ | | With $H_2O_2$ | |
|---|---|---|---|---|
| Steroid | K | $r^2$ | K | $r^2$ |
| $E_2$ | 0.0042[a] | 0.9691 | 0.0128[b] | 0.9843 |
| $E_1$ | 0.0043[a] | 0.8001 | 0.0110[b] | 0.9917 |
| $EE_2$ | 0.0045[a] | 0.8614 | 0.0144[b] | 0.9072 |
| T | — | — | 0.0041[a] | 0.8389 |

K: degradation rate constant.
[a]first-order rate constant ($min^{-1}$).
[b]zero-order rate constant ($\mu min \cdot^{-1}$).
$r^2$: coefficient of determination ($>0.8$).



hydrogen peroxide as described above. In spite of the presence of hydrogen peroxide, the same phenomenon has been observed as shown in Figure 3. These data prompted us to examine the photocatalytic degradation of T in the presence of hydrogen peroxide. T is one of the androgens and does not have a phenolic hydroxy group (Figure 1) and showed almost the same degradation pattern as that of $E_1S$. T was slowly degraded like $E_1$ or $E_2$ (without $H_2O_2$) by first order kinetics in the presence of hydrogen peroxide as shown in Table 3. It is interesting that hydrogen peroxide is ineffective for the photocatalytic degradation of $E_1S$ and $EE_23S$.

In order to obtain the intermediate of the photocatalytic degradation of $E_1S$ or related conjugates (such as $E_23S$ and $E_2$3-glucuronide[1]), we tried to detect the respective genin ($E_1$or $E_2$) or 9,11-dehydro-compound from the reaction mixture using HPLC, but the effort was fruitless. We hypothesized that the glucuronic acid (sugar) moiety on the estrogen skeleton plays an

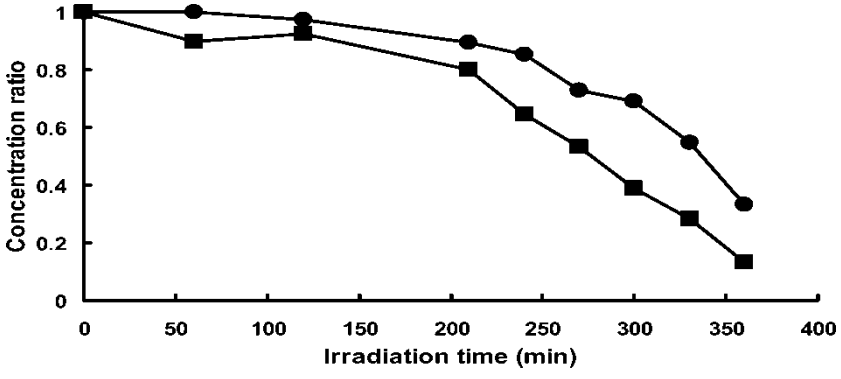


***Figure 3.*** Photocatalytic degradation of $E_1S$, ■ without $H_2O_2$, ● with $H_2O_2$.

important role in this degradation reaction as described in the previous paper.[1] We subjected Dt to this degradation reaction and about 40% of the Dt was gradually degraded within 6 hr. Dt is a cardiac glycoside having three digitoxoses and is metabolized to digitoxigenin by the stepwise degradation of three digitoxoses in the liver.[8] Dt-bis, having two digitoxoses, was obtained as the intermediate from the reaction mixture, which was confirmed by HPLC and LC/ESI-MS/MS in comparison with the authentic sample as shown in the experimental section. Dt does not contain a phenolic hydroxy group, so the above data suggests that the sugar moiety is important for this reaction (Figure 1).

Taoda *et al.* reported that the presence of $Fe^{3+}$ was effective for the degradation of organohalide compounds in a semiconductor suspension.[9] These data prompted us to examine the effect of $Fe^{3+}$ [$Fe(NO_3)_3 \cdot 9H_2O$, $4.95 \times 10^{-4}$ or $9.9 \times 10^{-4}$ M] or $Fe^{2+}$ [$FeSO_4 \cdot 7H_2O$, $4.95 \times 10^{-4}$M] on this photocatalytic degradation using $E_2$ as the substrate, but no apparent effect was observed.

The presence of hydrogen peroxide or $Fe^{3+}$ is considered to be effective for the creation of hydroxyl radicals and the other related radicals responsible for this photocatalytic degradation.[2,9]However, the above data showed that hydrogen peroxide is effective for the degradation of estrogen having an unconjugated phenolic hydroxy group such as $E_1$ and $E_2$, but $Fe^{3+}$ is not effective for this degradation.



## CONCLUSION

The monolithic silica column is effective for the fast separation of estrogens in these photocatalytic degradation experiments. Hydrogen peroxide is especially effective for the degradation of estrogen having an unconjugated phenolic hydroxy group such as $E_1$ or $E_2$, but not effective for that of $E_1$3S or $EE_2$3S having a sulfonic acid moiety at the phenolic hydroxy group. Much attention should be focused on these sulfates as endocrine disrupting chemicals.[10]

The intermediates of the photocatalytic degradation of $E_1$, $E_2$, and Dt were identified by comparison with authentic samples, but that of $E_1$S or the related conjugates was not obtained. Further experiments using HPLC with a monolithic silica column are now in progress in our laboratories to clarify this photocatalytic degradation mechanism.

## ACKNOWLEDGMENTS

Our thanks go to Drs. Honma (Teikoku Hormone Mfg.) and Kohtani (Kanazawa University) for supplying the substrates and authentic samples, respectively.

## REFERENCES


1. Mizuguchi, T.; Shibayama, Y.; Mitamura, K.; Shimada, K. Contribution of glucuronic acid and sulfonic acid moieties during photocatalytic degradation of estrogen conjugates. J. Health Sci. **2005**, *51* (4), 447–452.
2. Tanaka, K.; Hisanaga, T.; Harada, K. Photocatalytic degradation of organohalide compounds in semiconductor suspension with added hydrogen peroxide. New J. Chem. **1989**, *13* (1), 5–7.
3. Mizuguchi, T.; Ogasawara, C.; Shimada, K. Application of monolithic silica column for HPLC separation of estrogens. Chromatography **2005**, *26* (3), 101–104.
4. Tipson, R.S. Sulfonic Esters of Carbohydrates. Adv. Carbohydr. Chem. **1953** *8*, 107.
5. Palomino, E.; Heeg, M.J.; Horwitz, J.P.; Polin, L.; Brooks, S.C. Skeletal conformations and receptor binding of some 9,11-modified estradiols. J. Steroid Biochem. Molec. Biol. **1994**, *50* (1), 75–84.
6. Satoh, D.; Aoyama, K. Studies on digitalis glycosides XXXI. Stepwise degradation of polygitoxosides of cardenolides. Chem. Pharm. Bull. **1970**, *18* (1), 94–99.
7. Kohtani, S.; Kitajima, K.; Kudo, A.; Tokumura, K.; Nakgaki, R. $BiVO_4$ wo mochiita joseihormone no kashikou deno hikarishokubai bunkai, Spring Annual Meeting of Chemical Society of Japan, 2002, 2PA-035 (Tokyo) (in Japanese).
8. Doherty, J.E.; de Soyza, N.; Kane, J.J.; Bissett, J.K.; Murphy, M. Clinical pharmacokinetics of digitalis glycosides. Prog. Cardiovasc. Dis. **1978**, *21* (2), 141–158.
9. Taoda, H.; Watanabe, E.; Kato, K. Makujoushoukbai wo mochiita yuuki- ensokagoubutsu no hikarishokubaibunkai. Yousui to Haisui **1996**, *38* (4), 290–296 (in Japanese).
10. Reddy, S.; Iden, C.R.; Brownawell, B.J. Analysis of steroid conjugates in sewage influent and effluent by liquid chromatography-tandem mass spectrometry. Anal. Chem. **2005**, *77* (21), 7032–7038.


Received November 17, 2005
Accepted December 20, 2005
Manuscript 6761