Degradation of 17β-estradiol in aqueous solution by ozonation in the presence of manganese(II) and oxalic acid

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Available online: 01 May 2012
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(Received 6 July 2011; final version received 25 April 2012)

Natural estrogens, such as 17β-estradiol (E2), are the main substances responsible for estrogenic activity found in domestic sewage. In the work described herein, the degradation of E2 has been investigated by single ozonation and catalytic ozonation in the presence of manganese ion (Mn2+) and oxalic acid. The presence of Mn2+ and oxalic acid in the ozonation processes significantly improved the E2 degradation and, hence, the reduction of estrogenic activity in aqueous solution. The addition of Mn2+ and oxalic acid produced many more hydroxyl radicals in the catalytic ozonation system than in the single ozonation system. Oxidation products formed during ozonation of E2 have been identified by means of gas chromatography–mass spectrometry (GC–MS), on the basis of which a possible reaction pathway for E2 degradation by ozonation is proposed. E2 was first oxidized to hydroxyl-semiquinone isomers, and these were subsequently degraded to low molecular weight compounds such as oxalic acid and malonic acid. The latter were easily oxidized by ozone to form carbon dioxide (CO2).

The results demonstrate that the ozonation–Mn2+–oxalic acid system may serve as a powerful tool for removing E2, and the addition of Mn2+ and oxalic acid is favourable for the complete removal of estrogenic activity induced by steroid estrogens in aqueous solution.

Keywords: catalytic ozonation; 17β-estradiol (E2); hydroxyl radicals; manganese ion; degradation pathway

Introduction

Estrogen hormones in the environment have received increased attention because low concentrations (e.g. a few ng L⁻¹) of such hormones may have adverse effects on the normal activity of the endocrine system in both animals and humans [1,2]. Published studies have shown that these compounds can interfere with reproductive systems by producing an unnatural response in the endocrine system [3–5]. In particular, natural estrogens such as estrone (E1) and 17β-estradiol (E2), as well as synthetic estrogens such as 17α-ethynylestradiol (EE2) and levonorgestrel (LNG), have been found in the effluent of wastewater treatment plants at concentrations in the ng L⁻¹ range owing to incomplete removal during the wastewater treatment process, and are considered to be the major compounds responsible for observed endocrine-disrupting effects in wild fish. E2, excreted from animals and human beings, has been identified as having the highest estrogen-disrupting activity [6,7]. High levels of E2 of 150 ng L⁻¹ in raw domestic wastewater and 64 ng L⁻¹ in domestic wastewater treatment plant effluent have been reported [8,9]. A recent study demonstrated that exposure to E2 (>16 ng L⁻¹) affected the reproduction of male marine fish [10]. Furthermore, E2 resists degradation during the typical sewage treatment operation and is released into surface waters [11]. Therefore, it is very important and necessary to develop innovative treatment systems to deal with estrogens, especially E2.

In recent years, ozonation has been considered to be a promising technique owing to its high efficiency in removing these estrogen compounds [12,13]. Bila et al. reported that high E2 removal efficiencies (>99%) were achieved with low ozone dosages, and that estrogenic activity removal increased with ozone dosage but was not completely removed at pH 7 or 11 [14]. Irnak et al. showed that the time needed for complete conversion of 0.1 mmol of E2 was 55 min for an applied O3 dose of 15.78 × 10⁻³ mmol min⁻¹, and the intermediate products formed were determined [15]. A disadvantage of using ozonation alone for treating wastewater is that a large amount of energy is required for ozone generation. Moreover, full mineralization of estrogen is not achievable at the O3 doses commonly used in water treatment, and consequently biological activity is not completely removed since a variety of transformation products may be generated. Hence, it is important not only to identify the major initial transformation products, but also to assess their estrogenic activity relative to that of the parent compound.

In the last two decades, metal-catalytic homogeneous ozonation, combining a transition metal with ozone, has attracted considerable interest because of the need...
to improve ozonation efficiency and optimize economic efficiency. Several metal-catalytic ozonation systems, such as O$_3$-Mn(II), O$_3$-Fe(II), O$_3$-Zn(II), O$_3$-Co(II) and O$_3$-Ni(II), have been investigated [16–20]. The degradation efficiency of organics in catalytic ozonation systems exceeds that achieved by ozonation alone. Manganese salts have been used as catalysts for ozonation in numerous studies [21–24]. Ni et al. studied the ozonation of 2-chlorophenol solution in the presence of various kinds of metallic ions, including Pb$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Ti$^{2+}$ and Mn$^{2+}$ [25]. It was found that the reaction rate increased in all cases, and the best result was obtained by using Mn$^{2+}$. Ma and Graham reported that the oxidation of atrazine by ozone was greatly enhanced in the presence of a small amount of Mn(II) [26]. Xiao et al. demonstrated that a trace amount of Mn$^{2+}$ accelerated the mineralization of 2,4-dichlorophenol by ozone. Furthermore, several studies have indicated that metal ions such as Mn(II) or Mn(IV) form complexes with some organic compounds (e.g. oxalic acid), thereby increasing the rate of ozonation [27]. Xiao et al. reported that the addition of Mn$^{2+}$ coupled with oxalic acid accelerated the degradation of 2,4-dinitrotoluene (DNT) [28].

In the present study, E2 has been selected as a target compound, and its degradation efficiency and mechanisms in different ozonation systems (including ozonation alone, manganese-catalysed ozonation coupled with oxalic acid) have been investigated. The objectives of this study were: (i) to compare the degradation efficiency and estrogenic activity removal in several ozonation systems; (ii) to investigate the generation of hydroxyl radicals in several ozonation systems; (iii) to propose possible reaction mechanisms for E2 ozonation based on the by-products identified by GC-MS.

Materials and methods

Chemical reagents

17β-Estradiol (Sigma-Aldrich, >99% pure) was used as a model compound for catalytic ozonation. A stock solution of E2 at a concentration of 0.0368 mm was prepared by diluting an initial solution of 3.68 mm (1000 mg L$^{-1}$) in methanol, and was stored at 4°C. Ozone was produced with an ozone generator (CHYF-3A, rated flow 50 mg min$^{-1}$, Hangzhou Rongxin Electronic Equipment Co., Ltd, China), and stock solutions thereof were produced by sparging ozone-containing oxygen through Milli-Q water that was cooled in an ice bath. A 50 mg L$^{-1}$ manganese stock solution was prepared by dissolving MnSO$_4$·H$_2$O in deionized water. An internal standard, 17β-estradiol-d$_4$, purchased from Cambridge Isotope Laboratories (USA), was used to quantify the target compounds. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Sigma-Aldrich. All the organic solvents used, as well as 4-hydroxybenzoic acid, oxalic acid and sodium thiosulphate, were obtained from a local chemical company and used without further purification. All glassware was soaked in 10% HNO$_3$, then rinsed with tap water and deionized water.

Ozonation procedure

The catalytic ozonation experiments were carried out in 500 mL vials at room temperature (25°C). The aqueous solution consisted of Milli-Q purified water spiked with 10 μM E2 and adjusted to pH 7 with 10% HCl or NaOH. Manganese(II) and oxalic acid were introduced, and the reaction was initiated by ozone solution added under vigorous stirring. In this study, two different ozone concentrations (15 and 40 μM), coupled with Mn$^{2+}$ and oxalic acid, were selected. Samples (5 mL) of the reaction mixture were taken after the desired time intervals and immediately quenched with sodium thiosulphate. Samples were stored at 4°C and were analysed within 24 h.

Chemical analysis

Determination of E2 and intermediates

17β-Estradiol and intermediates in aqueous solution were extracted with dichloromethane, derivatized, and then analysed by GC-MS [29,30]. Briefly, the extracted liquid samples were spiked with 17β-estradiol-d$_4$ and then derivatized with pyridine and BSTFA. The derivatized samples were injected into a Hewlett-Packard (HP) 6890 gas chromatograph equipped with an HP 5-MS column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) and an HP 5975 mass-selective detector system. Helium carrier gas was maintained at a constant flow rate of 1.0 mL min$^{-1}$. The MS was operated in full-scan or selected-ion monitoring mode with positive ionization by electron impact. The inlet and MS transfer line temperatures were maintained at 280°C, and the ion source temperature was 250°C. Each sample (2 μL) was injected in splitless mode. To separate the compounds, the following GC column temperature programme was used: 100°C for 1 min, increased to 200°C by a ramp of 10°C min$^{-1}$ and to 300°C by a ramp of 3°C min$^{-1}$, and maintenance at 300°C for 10 min.

Analysis of dissolved ozone, Mn$^{2+}$ and hydroxyl radicals

Dissolved ozone in the aqueous phase was determined by the indigo method [31]. The concentration of Mn$^{2+}$ ions was determined using an inductively coupled plasma mass spectrophotometer (Agilent 7500a). Hydroxyl radicals react with 4-hydroxybenzoate (HDB) to produce 3,4-dihydroxybenzoate (DHDB), and the concentration of the latter was analysed by HPLC (Agilent Technologies 1200 Series) equipped with an Eclips XDB-C18 reversed-phase column (5 μm, 4.6 × 150 mm) and a UV detector (λ = 262 nm) [32]. The mobile phase buffer was a mixture of methanol and phosphoric acid (1 mL L$^{-1}$) (50:50, v/v).
The column was operated isocratically with a flow rate of 0.8 mL min\(^{-1}\), and was maintained at 30°C.

**Evaluation of estrogenic activities**

A two-hybrid yeast (Y187), containing estrogen receptor-\(\alpha\) (ER\(\alpha\)) and co-activator sequences, was constructed and was provided by the Research Centre for Eco-Environmental Science, China. Yeast cell culture and yeast assay were conducted as described by Gaido et al. [33] and Li et al. [34] with some modifications. The samples were serially diluted in dimethyl sulphoxide (DMSO). Aliquots (5 \(\mu\)L) from serial dilutions of test samples were combined with 995 \(\mu\)L of medium containing 5 \(\times\) 10\(^3\) yeast cells mL\(^{-1}\), resulting in a test culture. Aliquots (200 \(\mu\)L) of the test cultures were transferred to a 96-well plate and incubated at 30°C with vigorous orbital shaking (800 rpm) for 2 h. The cell density of the culture was then measured at 600 nm. Portions (50 \(\mu\)L) of the test culture were transferred to a new 96-well plate, 120 \(\mu\)L of Z-buffer (16.1 g L\(^{-1}\) Na\(_2\)HPO\(_4\) \(\cdot\) 7H\(_2\)O, 5.5 g L\(^{-1}\) NaH\(_2\)PO\(_4\) \(\cdot\) H\(_2\)O, 0.75 g L\(^{-1}\) KCl, 0.246 g L\(^{-1}\) MgSO\(_4\) \(\cdot\) 7H\(_2\)O) and 20 \(\mu\)L of chloroform were added, and the assay solutions were carefully mixed (vortexing for 1.5 min). The enzyme reaction was initiated by the addition of 40 \(\mu\)L of \(\alpha\)-nitrophenyl-\(\beta\)-D-galactopyranoside (13.3 mmol L\(^{-1}\), dissolved in Z-buffer) and incubated at 30°C for 60 min. The reactions were terminated by the addition of 100 \(\mu\)L of Na\(_2\)CO\(_3\) (1 mol L\(^{-1}\)). After centrifugation at 12,000 g for 15 min, 200 \(\mu\)L of the supernatant was transferred to a new 96-well plate, and the absorbance at 420 nm was measured. The \(\beta\)-galactosidase activity was calculated according to reports by Ma et al. [35].

**Results and discussion**

**E2 degradation in different ozonation systems**

Experiments were performed on different ozonation systems to investigate the degradation efficiency of E2, including ozonation alone, ozonation–Mn\(^{2+}\), and ozonation–Mn\(^{2+}\)–oxalic acid. Lee and von Gunten reported that 40 \(\mu\)M of ozone was depleted in less than 2 min, indicating low stability in solution [36]. Hence, a short reaction time was selected in these experiments.

Figure 1 presents the results of E2 degradation in the different ozonation systems. When either 15 or 40 \(\mu\)M ozone was added, the concentration of E2 decreased with increasing reaction time in each of the processes, that is, ozonation alone, ozonation–Mn\(^{2+}\), and ozonation–Mn\(^{2+}\)–oxalic acid. The efficiency of E2 degradation was higher in the presence of Mn\(^{2+}\) or Mn\(^{2+}\)–oxalic acid compared with the case of ozonation alone, and the highest E2 degradation efficiency was observed for ozonation with the simultaneous addition of Mn\(^{2+}\) and oxalic acid.

![Figure 1](image-url)
More than 90% of the E2 was degraded within 5 min by ozonation with the simultaneous addition of Mn\(^{2+}\) and oxalic acid in both the 15 and 40 \(\mu\)M ozonation systems. On the basis of the above results, it is clear that E2 was consumed very rapidly in the ozonation systems, and that E2 degradation was significantly promoted when Mn\(^{2+}\) and oxalic acid co-existed in the ozonation system.

**Estrogenic activity reduction in different ozonation systems**

In order to test whether estrogens could be transformed into non-estrogenic compounds by the ozonation systems, the estrogenic activities in solution were measured using a yeast two-hybrid system. Figure 2 shows the estrogenic activities remaining after 10 \(\mu\)M E2 was treated for 5 min in the 15 and 40 \(\mu\)M ozonation systems.

It can be seen in Figure 2 that the estrogenic activities in solution were decreased dramatically in the respective ozonation systems. When 15 \(\mu\)M O\(_3\) was added, the estrogenic activity was reduced to 1/8 by ozonation alone, to 1/18 by ozonation–Mn\(^{2+}\) and to 1/45 by ozonation–Mn\(^{2+}\)–oxalic acid, as expressed as a fraction of the initial activity (Figure 2a). At an ozone dose of 40 \(\mu\)M, the estrogenic activity was decreased to 1/16, 1/36, and 1/160 in the processes of ozonation alone, ozonation–Mn\(^{2+}\) and ozonation–Mn\(^{2+}\)–oxalic acid, respectively. This result suggested that the presence of Mn\(^{2+}\) and oxalic acid could increase the degree of mineralization of E2 in ozonation systems.

**\(\cdot\)OH production in different ozonation systems**

It is now widely accepted that ozone reacts in aqueous solution with various organic and inorganic compounds, either by direct selective reactions of molecular ozone or through a radical-type reaction involving hydroxyl radicals generated by the decomposition of ozone in water [37]. Because of the rapid decomposition of dissolved ozone in catalytic ozonation, it was necessary to determine whether many more hydroxyl radicals were produced in the presence of Mn\(^{2+}\) or Mn\(^{2+}\)–oxalic acid. The aromatic compound 4-hydroxybenzoic acid was used to trap \(\cdot\)OH, and the DHDB produced was quantified to assess the amount of hydroxyl radicals. It is shown in Figure 3 that the concentration of DHDB increased sharply within 2 min in the respective ozonation systems. Furthermore, compared with that in the case of ozonation alone, the concentration of DHDB was increased by 16% and 23% in the processes involving ozonation–Mn\(^{2+}\) and ozonation–Mn\(^{2+}\)–oxalic acid, respectively, which indicated that the presence of Mn\(^{2+}\) and oxalic acid favoured the additional production of hydroxyl radicals. Thus, the greater E2 degradation and reduction of estrogenic activity could be attributed to an increase of hydroxyl radical concentration produced by Mn\(^{2+}\) and oxalic acid.
Figure 3. Concentration of 3,4-dihydroxybenzoate (DHDB) in ozonation systems generating hydroxyl radicals. Conditions: O₃: 15 μM O₃ alone, O₃ + Mn²⁺: 15 μM O₃ coupled with 75 μM Mn²⁺, O₃ + Mn²⁺ + OA: ozonation catalysed by 15 μM O₃ coupled with 75 μM Mn²⁺ in the presence of 230 μM oxalic acid. T = 25 °C, initial pH 7.0, initial E₂ concentration of 10 μM.

Figure 4. Concentration of dissolved manganese in ozonation systems. Conditions: O₃ + Mn²⁺ + OA: ozonation catalysed by 40 μM O₃ coupled with 200 μM Mn²⁺ in the presence of 611 μM oxalic acid. Conditions: T = 25 °C, initial pH 7.0, initial E₂ concentration of 10 μM.

**Analysis of intermediates**

**Mn²⁺ concentration in solution**

As previously assumed, Mn must exist in ionic form for the sustainable production of hydroxyl radicals in solution. As shown in Figure 4, the concentration of dissolved manganese ions decreased slightly within 2 min, but then remained constant within 20 min in the ozonation systems, which was consistent with the reduction of E₂. This can be rationalized in terms of the catalytic mechanism proposed by Xiao *et al.* [28]. In the ozonation systems, Mn²⁺ catalysed the decomposition of ozone to generate hydroxyl radicals, and at the same time the Mn²⁺ was oxidized to higher-valent manganese oxide. Pollutants were degraded by reaction with hydroxyl radicals, and oxalic acid reduced manganese oxide to lower-valent Mn²⁺:

\[
\text{Mn}^{2+} + \text{O}_3 + \text{H}^+ \rightarrow \text{Mn}^{3+} + \cdot \text{OH} + \text{O}_2
\]

\[
2\text{Mn}^{3+} + 2\text{H}_2\text{O} \rightarrow \text{Mn}^{2+} + \text{MnO}_2 + 4\text{H}^+
\]

\[
\cdot \text{OH} + \text{organic} \rightarrow \text{intermediates}
\]

\[
\text{MnO}_2 + \text{H}_2\text{C}_2\text{O}_4 + 2\text{H}^+ \rightarrow \text{Mn}^{2+} + 2\text{CO}_2 + 2\text{H}_2\text{O}
\]

\[
\text{Mn}^{2+} + \text{O}_3 + \text{H}^+ \rightarrow \text{Mn}^{3+} + \cdot \text{OH} + \text{O}_2
\]

**Identification of intermediates in E₂ ozonation**

To elucidate the structures of the intermediates, the samples obtained after 2 min in single ozonation and Mn²⁺–oxalic acid catalytic ozonation were subjected to GC-MS analysis. In single ozonation, many organic intermediates were produced, including 2-hydroxyl-E₂, E₂ semiquinone, oxalic acid, and malonic acid, as shown in Table 1. In contrast, these intermediates were not found in the case of Mn²⁺–oxalic acid catalytic ozonation.

**Proposed reaction pathway**

Based on analysis of the intermediates, it is suggested that E₂ underwent ozonation in the case of single ozonation. The frontier densities (FEDs) for the E₂ molecule have been evaluated by Ohko *et al.* [38], and it was found that

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Intermediates</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.45</td>
<td>Estradiol semiquinone</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>14.08</td>
<td>2-Hydroxyl-estradiol</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>10.55</td>
<td>Oxalic acid</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>12.50</td>
<td>Malonic acid</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 5. The possible reaction pathway for E2 oxidation by ozone.

the $2\text{FED}^2_{\text{HOMO}}$ was higher at the phenol moiety, especially at the C10 and C3 atoms (the values were 0.508 and 0.364, respectively). So, the C10 or C3 atoms should be the sites at which the first electron is extracted. The reaction may be initiated by $\cdot \text{OH}$ at C2 and C5 on the basis of $\text{FED}^2_{\text{HOMO}} + \text{FED}^2_{\text{LOMO}}$ values. Referring to published work [39], a probable pathway for the ozonation of E2 is proposed in Figure 5.

The first addition of an OH radical occurs at the C2 atom, thus producing the corresponding 2-hydroxyl-E2 radical. The attack of $\cdot \text{OH}$ was followed by a dehydration reaction, yielding E2 semiquinone radical as a resonance structure.
E2 semiquinone could then be produced by direct attack of •OH at the C10 or C2 atom of the resonance structure. Subsequently, further oxidation led to breakdown of the aromatic structures, leading to organic acids and ultimately to carbon dioxide.

Conclusion
The catalytic ozonation of E2 in aqueous solution has been studied in the presence of Mn2+ and oxalic acid. Under the experimental conditions, the addition of Mn2+ and oxalic acid significantly promoted E2 degradation and the reduction of estrogenic activity. Furthermore, the presence of Mn2+ and oxalic acid favoured the additional production of hydroxyl radicals, which accelerated E2 degradation.

The concentrations of Mn2+ and organic intermediates were determined in the respective ozonation systems. Manganese maintained dissolved ion status in the catalytic ozonation, and was the main catalyst of E2 ozonation. Some organic intermediates were produced in the ozonation of E2, and the main products were semiquinones, which were further oxidized to organic acids of lower molecular weight.

Acknowledgements
This study was sponsored by the National Natural Science Foundation of China (Grant No. 21107097) and the Natural Science Foundation of Zhejiang Province (Grant No. Y5110118).

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