Environmental Engineering Research

Environ. Eng. Res. 2020
Short Communication
https://doi.org/10.4491/eer.2019.270
pISSN 1226-1025 eISSN 2005-968X
In Press, Uncorrected Proof

Comparison of biological and chemical assays for measuring the concentration of residual antibiotics after treatment with gamma irradiation

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Abstract

Antibiotic pollution is one of the factors contributing to the spread of antibiotic-resistant bacteria in the environment. Advanced oxidation and irradiation processes have been introduced to eliminate antibiotics from water and wastewater. However, few studies have reported the toxic effects of residual antibiotics and their byproducts induced by a treatment system. In this study, we compared the efficacies of chemical (high-performance liquid chromatography [HPLC]) and biological (antimicrobial susceptibility test) assays for measuring the concentrations of residual antibiotics after gamma irradiation for degrading amoxicillin, cephradine, lincomycin, and tetracycline. The concentrations of residual antibiotics estimated using the two assay methods were almost identical, except cephradine. In the case of cephradine, inhibited bacterial growth was observed that was equivalent to twice the concentration measured by HPLC in the samples subjected to gamma irradiation. The observed inhibition of bacterial growth suggested the generation of potentially toxic intermediates following antibiotic degradation. These results indicate that biological and chemical assays should be used in concert for monitoring antibiotic contamination and the toxic derivatives of antibiotic degradation. The results demonstrate that these four antibiotics can be decomposed by 2.0 kGy gamma-irradiation without toxic effects of their byproducts.

Keywords: Antibiotics, Antimicrobial susceptibility test, Chemical assay, Gamma irradiation

Received June 24, 2019 **Accepted** August 4, 2019

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1. Introduction

Antibiotics are powerful medicines used to treat infections caused by microorganisms. However, the inappropriate use of antibiotics and their proliferation in the environment can cause toxic effects in aquatic organisms [1]. Studies have indicated that wastewater treatment facilities are one of the important point sources for antibiotic contamination of surface waters [2-5]. The antibiotics in the final effluents released from these facilities are presumed to the reason for the spread of antibiotic-resistant bacteria in the environment [6-8].

Degradation of antibiotics via biological processes has been investigated to treat wastewater containing antibiotics. However, many studies have demonstrated that clinically important antibiotics are not completely biodegraded by conventional treatment methods, even when employing a combined anaerobic–aerobic treatment system, which has been used to treat high-strength pharmaceutical wastewater [9-11]. Advanced oxidation processes (AOPs), such as hydrogen peroxide (H_2O_2) or ozone (O_3), catalysts (iron ions, electrodes, and metal oxides), and irradiation (UV, sunlight, ultrasound, and gamma irradiation) have shown potential as alternative processes for the treatment of most industrial effluents containing toxic organic chemicals [12-19]. Ozonation can be successfully employed as a pretreatment to enhance biodegradability of antibiotics in wastewater, although not for complete mineralization of the antibiotic

(>90% removal efficiencies) [13, 16, 20-22]. Removal rates have been reported to be 98% for tetracycline when used in combination with UV and TiO_2 as a catalyst, while degradation of lincomycin was noticeably lower [22, 23]. The UV/TiO_2 treatment also degrades 82% of the sulfamethoxazole [24]. The occurrence of antibiotics in the effluents of wastewater treatment facilities supports concerns regarding discharged antibiotic residues that may reside in the water supply, and thus have potentially serious environmental consequences [25]. When original medicinal modes of action disappear, degradation products should not promote formation of resistant bacterial strains [22, 26]. However, degradation compounds must be identified and monitored, as they may be more toxic than the parent compounds [22, 27].

Liquid chromatography coupled with mass spectrometry or tandem mass spectrometry has been routinely used to measure antibiotics in wastewater, and these techniques are assumed to be sufficiently accurate and sensitive to detect these compounds [14, 28-30]. However, analytical methods require time-consuming extraction and concentration steps to prepare samples and are not suitable for detecting derivatives resulting from partial biodegradation, which have the potential to induce microbial resistance to antibiotics and affect the environment [31]. Cephalosporin antibiotics have been detected using high-performance liquid chromatography (HPLC), and the toxicity of the residual compound by direct and indirect photolysis has been measured using the

Microtox test [32]. Similarly, Li et al. [33] reported on a toxic byproduct of oxytetracycline that was generated by ozone treatment, and which affected aquatic microbial activity as measured by a bioluminescence assay using *Vibrio fischeri*.

As an alternative to chemical methods, a bioassay has been introduced to detect residual antibiotics in wastewater. Using these methods, resistance to antibiotics and antibiotic toxicity has been estimated by measuring the extent of specific gene expression [34, 35] or the inhibition of microbial activity [30, 31]. However, these methods display a relatively lower sensitivity than HPLC assays. This lower sensitivity, along with the presence of various organic compounds and their degradation intermediates in pharmaceutical wastewaters, makes such biological methods unsuitable for estimating antibiotic toxicity [30].

Gamma irradiation has been shown to be effective for promoting the complete decomposition and mineralization of antibiotics (amoxicillin, cefaclor, cephradine, tetracycline, lincomycin, and sulfamethazine) [18, 19, 36]. Gamma irradiation is a more efficient and economical treatment method than other AOPs [17, 36]. Gamma irradiation using a ^{60}Co source produces radiolysis of water, resulting in the production of radicals, such as oxidizing ($^{\bullet}OH$) and reducing species (e_{aq}^{-}, $H^{\bullet}$). These radicals are thought to play a major role in antibiotic degradation [36]. However, few studies have reported the toxic effects of residual antibiotics and their byproducts induced by gamma irradiation.

58 The present study investigated degradation of antibiotics, such as amoxicillin
59 (penicillins), cephradine (cephalosporins), lincomycin (lincosamides), and tetracycline
60 (tetracyclines) using gamma irradiation. Additionally, we propose a dilution method as a
61 bioassay for estimating the toxicity of residual antibiotics and their degradation
62 intermediates, and we evaluate the efficacy of this method for monitoring antibiotic-
63 containing wastewater treated with gamma irradiation by comparing it with a routine
64 HPLC assay.
65

66 ## 2. Experimental

67 ### 2.1. Cultures and Reagents

68 The four antibiotics used in this study, such as amoxicillin, cephradine, lincomycin, and
69 tetracycline, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) (Table S1).
70 The antibiotics were prepared for gamma irradiation by dissolving them in distilled
71 water at a concentration of 30 mg/L. Two bacterial strains, *Staphylococcus aureus*
72 KCTC 1621 (ATCC 25923) and *Escherichia coli* KCTC 1682 (ATCC 25922), were
73 purchased from the Korean Collection for Type Cultures (Daejeon, Korea), and
74 incubated in Mueller-Hinton broth (Difco, Sparks, MD, USA) [37].
75

76 ### 2.2. Gamma Irradiation

77 Gamma irradiation was produced using a high-level ^{60}Co source (Nordion Inc., Laval,
78 QC, Canada) at the Korea Atomic Energy Research Institute (Jeongeup, Korea). The
79 radioactivity of the source was approximately 1.47×10^{17} Bq (= 397,949 Ci), with dose
80 rates ranging from 6.3 to 14.3 kGy/h, depending on the distance from the source (up to
81 100 kGy). The absorbed doses were measured using the alanine-EPR dosimetry system
82 in accordance with ISO/ASTM 51607:2004 [38]. Aqueous sample solutions containing
83 antibiotics were placed into 50 mL conical tubes without any headspace for gamma
84 radiolysis. All solutions were equilibrated at atmospheric pressure and room temperature
85 (22 ± 2℃) before being irradiated, and were subsequently sealed with screw caps to
86 prevent introduction of air.
87
88 **2.3. Chemical Assay**
89 The concentrations of antibiotics in the aqueous samples were determined by HPLC,
90 using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA,
91 USA) equipped with a UV absorbance detector operated at 230, 254, 355, and 210 nm
92 for amoxicillin, cephradine, tetracycline, and lincomycin, respectively. The analytical
93 methods used for each antibiotic are summarized in Table 1. Triplicate subsamples were
94 prepared and analyzed for each sample.
95 **Table 1**

96 To analyze the mass profile of the cephradine degradation products generated by gamma
97 irradiation, the assay was performed using an Agilent 1100 module (Agilent, Palo Alto,
98 CA, USA) equipped with a Luna C18 column (150 mm × 2.0 mm, i.d.: 3 µm;
99 Phenomenex, Torrance, CA, USA). The flow rate was set to 0.15 mL/min, and injection
100 volume was 5 µL. A mixture of acetic acid (0.5% v/v) and methanol (42:58, v/v) was
101 used as the mobile phase. All target compounds were eluted out of the column within 15
102 min. The auto-sampler temperature was operated at 10℃. Mass spectrometric
103 measurements were carried out on a Sciex API 3000 triple-quadrupole tandem mass
104 spectrometry (Applied Biosystems, Foster City, CA, USA) equipped with an
105 electrospray ionization (ESI) interface in positive mode for cephradine and byproducts.
106 Ions were acquired in multiple reaction monitoring mode with a dwell time of 10 ms.
107 The mass spectrometer conditions were as follows: ion spray voltage: 5.5 kV, curtain gas:
108 10 L/min, nebulizer gas: 5 L/min, Auxiliary gas: 6.1 L/min, heated capillary temperature:
109 300℃, interface heater: ON, and collision gas: 5.
110
111 **2.4. Biological Assay**
112 To measure the minimal inhibitory concentration (MIC) of the antibiotics, we used the
113 antimicrobial susceptibility (AMS) test described by Jorgensen and Hindler [39]. Briefly,
114 120 µL of serially diluted antibiotic was dispensed into the wells of a 96-well microplate,

and these dilutions were subsequently inoculated with 60 μL of the bacterial strains in Mueller-Hinton broth. The final number of *Staphylococcus aureus* or *Escherichia coli* in the reaction mixtures was approximately 1.0×10^5 CFU/mL. After inoculation, the microplates were incubated in a shaking incubator for 18-20 h at 37℃. Bacterial growth was measured after the incubation using a microplate reader (ELx800; BioTek, Winooski, VT, USA) at a wavelength of 595 nm. The minimum concentration of antibiotic, at which over 95% of bacterial growth was inhibited, was considered the MIC. The concentrations of residual antibiotics in the samples after gamma irradiation were estimated using the AMS test and MIC of the antibiotics. The samples were serially diluted and inoculated with the bacterial strains at a final concentration of 1.0×10^5 CFU/mL. After measuring bacterial growth, antibiotic concentration was calculated by multiplying the MIC of the relevant antibiotic by the dilution factor at which bacterial growth was inhibited. If the survival rates of two consecutively diluted samples decreased significantly, then the dilution range was subdivided and the growth of bacteria was re-measured to precisely estimate the inhibition range. All experiments were performed in triplicate.

3. Results and Discussion

3.1. Minimal Inhibitory Concentration of Antibiotics for the Test Strains

134 The antibiotics used in this study were differentiated into two groups based on their
135 mechanisms of action: β-lactam antibiotics, such as amoxicillin and cephradine, inhibit
136 bacterial cell wall synthesis, whereas non β-lactam antibiotics, such as lincomycin and
137 tetracycline, inhibit protein synthesis [40]. The inhibitory effects of the different
138 concentrations of antibiotics on growth of the test strains are shown in Fig. 1. Among the
139 antibiotics examined, tetracycline was the most effective at inhibiting the growth of both
140 test organisms, and the MICs for *E. coli* and *S. aureus* were ≤ 0.625 and ≤ 0.109 mg/L,
141 respectively. The MICs determined using *S. aureus* were significantly lower for the other
142 antibiotics (amoxicillin, cephradine, and lincomycin) than those determined using *E. coli*.
143 The MIC value of amoxicillin, cephradine, and lincomycin against *S. aureus* were ≤
144 0.125, ≤ 1.75, and ≤ 0.109 mg/L, respectively. *E. coli* exhibited high resistance to
145 amoxicillin and cephradine with MIC values ≤ 10.00 and ≤ 16.00 mg/L, respectively.
146 The MIC value of lincomycin for *E. coli* was > 30 mg/L. These results indicate that *S.*
147 *aureus* was more sensitive than *E. coli* to each of the antibiotics examined.
148 (a) (b)

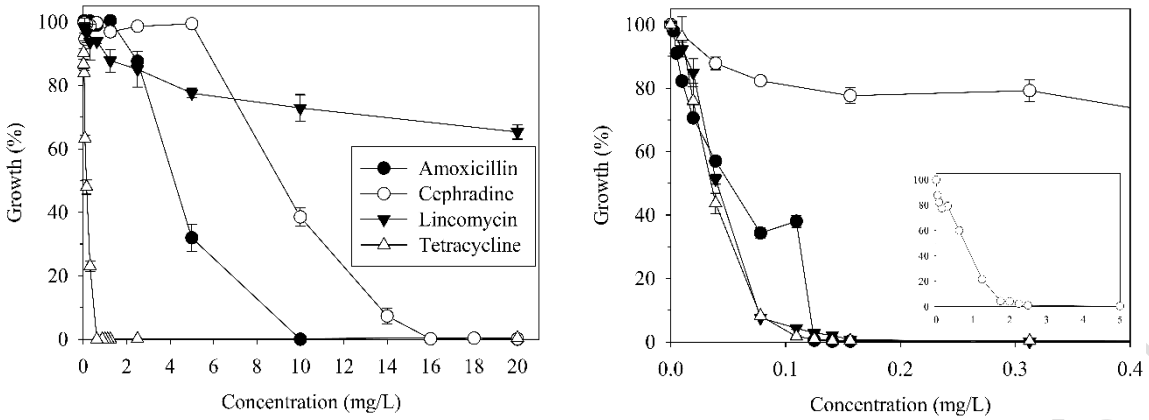
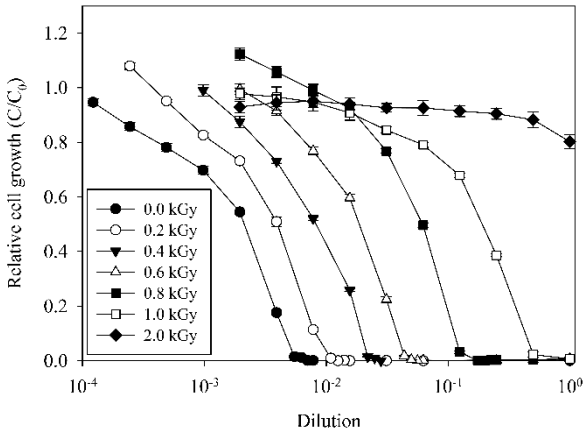


Fig. 1. Growth curve of *Escherichia coli* KCTC 1682 (a) and *Staphylococcus aureus* KCTC 1621 (b) at various concentrations of amoxicillin, cephradine, lincomycin, and tetracycline. Cell growth (%) was calculated as the $OD_{595\ nm}$ value of the antibiotic sample was divided by the $OD_{595\ nm}$ value of the blank (distilled water). Hydrophilic antibiotics (amoxicillin, cephradine, and tetracycline) pass more easily through pore-forming porins compared to hydrophobic antibiotics (lincomycin), which diffuse across the lipid bilayer [41, 42]. Resistance to hydrophobic antibiotics in Gram-negative bacteria may be either due to a decrease in penetration of the antibiotic through the outer membrane or due to specific mechanisms, such as a gene mutation or acquisition of resistance genes [41, 43]. Moreover, Gram-negative bacteria are generally more readily resistant to antibiotic compounds because their outer membrane protects the peptidoglycans [41, 42]. Most β-lactam antibiotics, including amoxicillin and cephradine, work by inhibiting cell wall biosynthesis in bacteria and are mainly active against Gram-

163 positive bacteria, such as *S. aureus.* Therefore, the results suggest that *S. aureus* is
164 suitable for estimating the residual concentrations of these antibiotics.
165
166 **3.2. Concentrations of Residual Antibiotics after Treatment with Gamma**
167 **Irradiation**
168 The residual concentrations of the antibiotics in the samples treated with gamma
169 irradiation were measured using both biological and chemical methods. In the biological
170 assay, the samples treated with up to 2.0 kGy of gamma irradiation were serially diluted,
171 and the growth of *S. aureus* was observed (Fig. 2). Using the dilution factor at which
172 growth of the strain was completely inhibited (≥ 95%) and the MIC of the relevant
173 antibiotic for *S. aureus*, we calculated the residual bioactive concentrations.
174 (a) (b)



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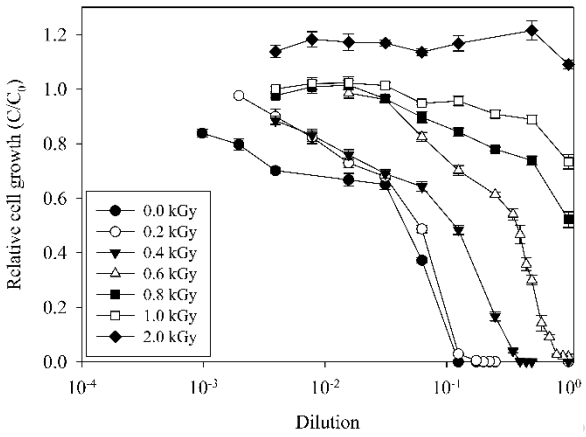

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178 (c) (d)

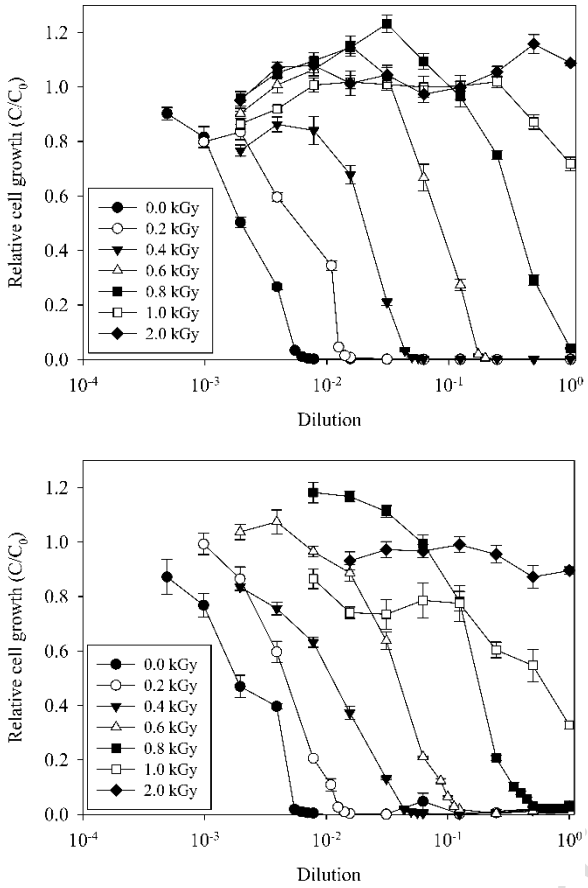


Fig. 2. Survival rates of *Staphylococcus aureus* KCTC 1621 in samples of amoxicillin (a), cephradine (b), lincomycin (c), and tetracycline (d) treated with 0–2 kGy of gamma irradiation. Relative cell growth (C/C_0) was calculated as the $OD_{595\ nm}$ value of the sample was divided by the $OD_{595\ nm}$ value of the blank (distilled water).Inactivation of the antibiotics was directly proportional to the strength of gamma irradiation, as observed previously [18, 19, 44]. The four antibiotics used in this study at an initial concentration of 30 mg/L were completely inactivated by the 2.0 kGy gamma irradiation treatment (Fig. S1). With the exception of cephradine, the residual

concentrations of antibiotics estimated using HPLC and the AMS test were not significantly different (Table 2). In the case of cephradine, the residual concentrations in the samples treated with 0.2-0.6 kGy gamma irradiation were significantly different between the two methods (paired *t*-test, $p < 0.04$), whereas the values for the samples treated with irradiation greater than 1.0 kGy were similar. The residual concentrations of cephradine treated with 0.2 kGy gamma irradiation were 23.56 ± 0.00 and 14.27 ± 0.02 mg/L as determined by the AMS test and HPLC assay, respectively (Table 2). The corresponding values for the samples treated with 0.6 kGy were 3.68 ± 0.00 and 1.49 ± 0.03 mg/L. This discrepancy between the two methods may be attributable to the partially decomposed byproducts of cephradine generated by the gamma irradiation, and these byproducts are capable of inhibiting bacterial growth. These results suggest the potential risk of increased eco-toxicity from cephradine after exposure to gamma irradiation treatment.

The byproducts of cefazolin, cephapirin, cephalexin, and cephradine are toxic according to the Microtox test [32]. Hafkemeyer et al. [45] reported that a degradation product of ceftazidim, which is a third-generation cephalosporin antibiotic, exhibits an inhibitory effect against RNase H. The degradation product of cefazolin, a first-generation cephalosporin antibiotic, was identified as a primary toxic byproduct using the zebrafish embryo toxicity test [46]. These cases provide indirect evidence that the

209 degradation products (byproducts) of cephalosporin antibiotics are probably “bioactive”
210 substances.
211
212 **3.3. Decomposition of Cephradine by the Gamma Irradiation Treatment**
213 Two distinct peaks with retention times of 3.10 and 6.17 min were detected in the LC
214 chromatograms of cephradine, which accounted for the very lower portion than the main
215 peak (7.16 min) in the chromatograms of untreated cephradine (Fig. 3). When the
216 irradiation dose increased to 0.6 kGy, the peak area of 6.17 min increased to 72.4% of
217 the area of 7.16 min and the peak with a retention time of 6.17 min disappeared above 1
218 kGy. Other studies have shown that 30 mg/L of antibiotics (e.g., amoxicillin, cefaclor,
219 cephradine, tetracycline, lincomycin, and sulfamethazine) were completely degraded and
220 mineralized after 1 kGy gamma radiation [18, 19, 36]. These findings suggest that the
221 peak at 6.17 min was a “bioactive” substance and a microbial activity inhibitor (Fig. 3B
222 and Table 2). In contrast, the area of the peak at 3.10 min continually increased with
223 increasing strength of gamma rays (Fig. 3C). These results indicate that the compound
224 showing a peak at 3.10 min was a degradation product of gamma irradiation and was not
225 an inhibitor of bacterial growth (Table 2).
226 The gamma radiolysis of water generates some active species as given Eq. (1)
227 [47].

$$H_2O \rightarrow {}^{\bullet}OH(2.7) + e_{aq}^{-}(2.6) + H^{\bullet}(0.55) + H_2(0.45) + H_2O_2(0.71) + H_3O^{+}(2.6) \quad (1)$$

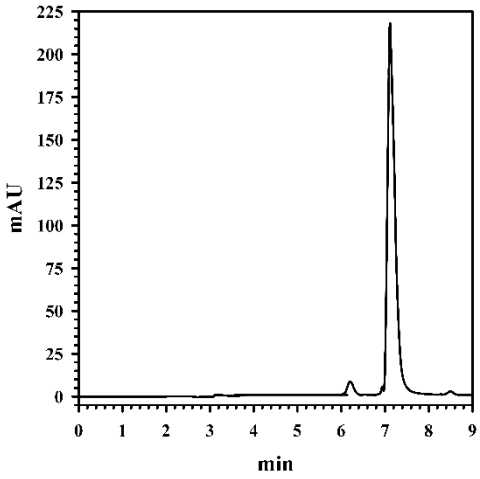
The values in parenthesis are the average radiation chemical yield (G-value), which was defined as the number of product molecules formed per 100 eV absorbed at pH 6.0-8.5 [47]. Hydroxyl radicals ($^{\bullet}OH$) and hydrated electrons (e_{aq}^{-}) are two main reactive species. It is known that $^{\bullet}OH$ radical is powerful oxidants, nonselective and highly reactive with organic matter, while e_{aq}^{-} is a strong reducing agent. The gamma irradiation may degrade antibiotics through oxidation and reduction pathway [47]. As shown in Fig. 3F and Table 2, absorbed gamma irradiation doses in the range of 0.2-0.6 kGy caused a dose-dependent degradation of the antibiotic and a decrease in cephradine (7.16 min, $[M+H]^+ = 350$) content due to radiation conversion to byproduct (6.17 min, $[M+H]^+ = 348$). Wang and Lin [32] reported that cephradine ($[M+H]^+ = 350$) reacted immediately with $^{\bullet}OH$ radicals and were transformed cephalexin ($[M+H]^+ = 348$). Therefore, the gamma radiolysis route results in the formation of cephalexin and later formed byproducts [48, 49]. Moreover, both cephradine and cephalexin are β-lactam antibiotics and within the class of first-generation cephalosporins. The structurally similar cephradine and cephalexin exhibited similar levels of toxicity after irradiation. López Peñalver et al. [50] evaluated toxicity during the irradiation-induced degradation of tetracycline in an aquatic environment using the *Vibrio fischeri* inhibition test. Similar to our results, they revealed that toxicity increased at 0.1-0.4 kGy possibly due to the

247 production of more toxic byproducts, which decreased when the dose was increased to
248 1.0 kGy. Our studies also demonstrate that the four antibiotics can be decomposed by 2.0
249 kGy gamma irradiation without toxic effect of their byproducts.

250 Although degradation of the original drug is readily achieved in wastewater
251 treatment systems (e.g., chlorination, ozonation, and AOPs), the byproducts generated
252 can be less biodegradable, have similar biological activity, and/or more be toxic than the
253 parent compound [12, 25, 32]. For example, Dantas et al. [51] observed a slight increase
254 in acute toxicity during the first-stage of ozonation of sulfamethoxazole using the
255 Microtox test. Alsager et al. [12] also tested the biological activity of ozone-treated
256 antibiotics with a well-established *E. coli* test. The synergistic effect of toxicity caused
257 by intermediate products or byproducts of antibiotics cannot be ignored and warrants
258 future research [32, 47]. Wang et al. [52] reported that the toxicity of intermediate
259 products or byproducts of antibiotics after ionizing irradiation (gamma ray and electron
260 beam) are significant when evaluating the potential dangers to human and ecological
261 systems. They also suggested that more studies should be conducted to explore bio-
262 toxicity.

263

264 (a) (d)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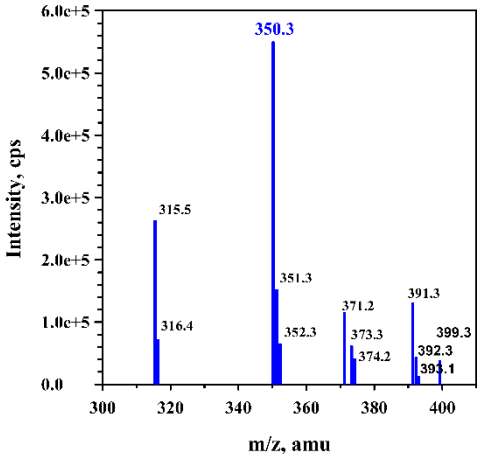
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350.3
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316.4
351.3
352.3
371.2
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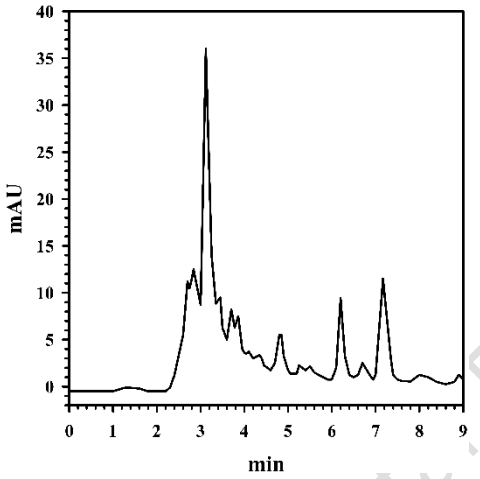
391.3
392.3
393.1
399.3

(b) (e)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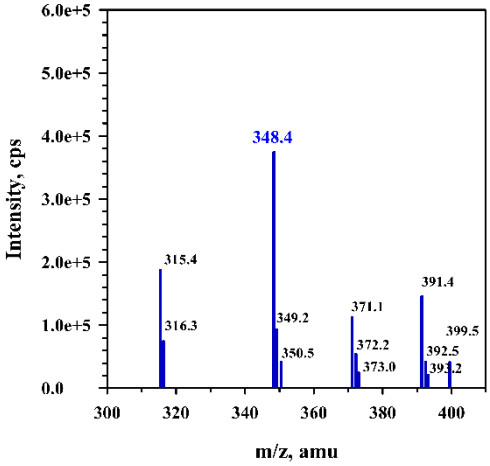
Intensity, cps
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315.4
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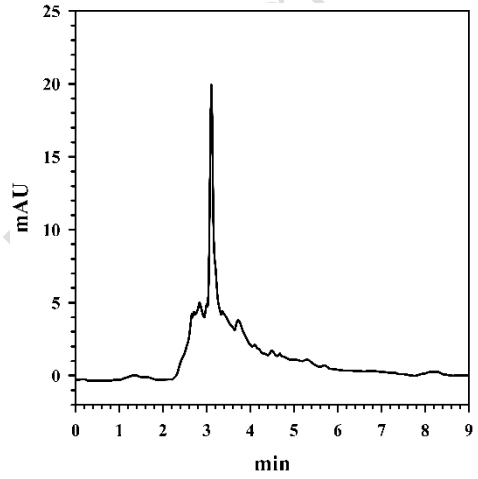
(c) (f)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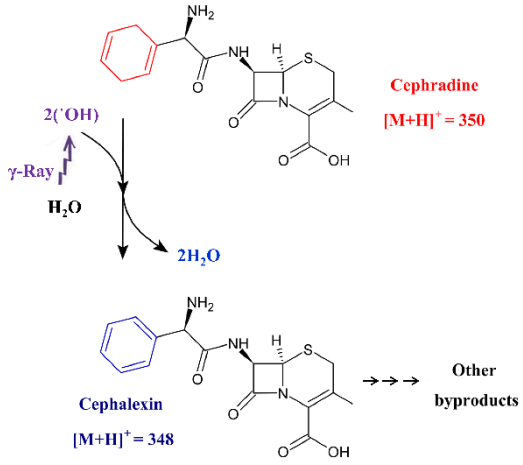
Cephradine
[M+H]+ = 350
2(·OH)
γ-Ray
H2O
2H2O
Cephalexin
[M+H]+ = 348
Other
byproducts

Fig. 3. Chromatograms of the liquid chromatography analysis of untreated cephradine (a) and of cephradine treated with 0.6 kGy (b) and 1.0 kGy (c) gamma irradiation. Mass chromatograms of cephradine (d, Retention time of 7.16 min in (a) and (b)) and byproduct (e, Retention time of 6.17 min in (b)). Predicted gamma radiolysis products and degradation pathway of cephradine (f). The byproducts were detected in positive mode as $[M+H]^+$.

Table 2

4. Conclusions

Although the measurement sensitivities of our two assay methods were almost identical, the AMS biological assay detected toxic antibiotic derivatives that were not detected by HPLC. The results presented in this study suggest that the AMS biological assay may be more useful than the HPLC chemical assay for measuring bioactive residual antibiotics in environmental samples. Therefore, we suggest that the chemical assay should be used in parallel with the bioassay for measuring antibiotics and their byproducts. It is also necessary to use a sufficiently strong irradiation dose to ensure that potentially toxic byproducts are completely degraded and do not affect the ecosystem when using gamma irradiation to treat environmental samples containing antibiotics.

290 ## Acknowledgements

291 This work was supported by Nuclear Research & Development Program through an
292 NRF grant funded by the Ministry of Science and ICT, Korea.

293

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433 s by ionizing radiation: an overview. *Sci. Total Environ.* 2019;646:1385-1397.
434

435 **Table 1.** Details of the High-performance Liquid Chromatography Methods Used to

436 Analyze the Antibiotics

Antibiotics	Column stationary phase	Injection volume (µL)	Flow rate (mL/min)	UV detection (nm)	Eluent
Amoxicillin	C8[a]	50	1.0	230	25 mM Potassium phosphate (pH 4.6):Methanol (95:5)
Cephradine	Polar RP[b]	50	1.0	254	20 mM Ammonium formate (pH 3.5):Methanol (65:35)
Tetracycline	C8[a]	50	1.0	355	10 mM Oxalic acid (pH 2.0):Methanol:Acetonitrile (72:8:20)
Lincomycin	C18[c]	75	0.7	210	1 mM Ammonium formate (pH 9.0):Acetonitrile (65:35)

437 [a]Luna 5 µ C8(2) 100A 150 × 4.6 mm (Phenomenex, Torrance, CA, USA)

438 [b]Synergi 4 µ Polar-RP column 150 × 4.6 mm (Phenomenex)

439 [c]Zorbax SB-C18 250 × 3.0 mm (Agilent Technologies, Santa Clara, CA, USA)

Table 2. The Residual Concentrations of Antibiotics in the Samples Treated with Gamma Irradiation, Estimated Using High Performance Liquid Chromatography (HPLC) and the Antimicrobial Susceptibility (AMS) Test

Antibiotics (mg/L) / **Gamma irradiation (kGy)**	**Amoxicillin**		**Cephradine**		**Lincomycin**		**Tetracycline**	
	HPLC	**AMS[a]**	**HPLC**	**AMS**	**HPLC**	**AMS**	**HPLC**	**AMS**
0.0	31.16 (± 0.03)[b]	27.94 (± 0.00)	29.72 (± 0.02)	23.56 (± 0.00)	31.63 (± 0.90)	33.43 (± 0.00)	28.10 (± 0.51)	31.07 (± 0.00)
0.2	15.11 (± 0.15)	13.97 (± 0.00)	14.27 (± 0.23)	23.56 (± 0.00)	12.61 (± 0.03)	14.63 (± 0.00)	11.46 (± 0.04)	13.59 (± 0.00)
0.4	5.87 (± 0.04)	6.98 (± 0.00)	4.74 (± 0.01)	8.41 (± 0.00)	4.02 (± 0.03)	4.18 (± 0.25)	3.36 (± 0.04)	3.65 (± 0.23)
0.6	2.71 (± 0.01)	3.49 (± 0.00)	1.49 (± 0.03)	3.68 (± 0.00)	1.83 (± 0.05)	1.01 (± 0.03)	1.12 (± 0.01)	1.56 (± 0.05)

0.8	0.76 (± 0.03)	1.22 (± 0.00)	0.13 (± 0.00)	< 2.94	0.74 (± 0.01)	0.18 (± 0.00)	0.21 (± 0.00)	0.35 (± 0.01)
1.0	0.17 (± 0.01)	0.19 (± 0.00)	ND	< 2.94	ND	< 0.18	0.03 (± 0.00)	< 0.17
2.0	ND[c]	< 0.15	ND	< 2.94	ND	< 0.18	ND	< 0.17

[a] The residual concentrations measured by AMS test were estimated by the toxicity of residual parent antibiotics and byproducts.

[b] Numbers in parentheses indicate the standard deviation of three replicates.

[c] ND, below the detection limit (amoxicillin: 0.03 mg/L, cephradine: 0.03 mg/L, lincomycin: 0.05 mg/L, tetracycline: 0.01 mg/L