^{60}Co-Irradiation as an Alternate Method for Sterilization of Penicillin G, Neomycin, Novobiocin, and Dihydrostreptomycin

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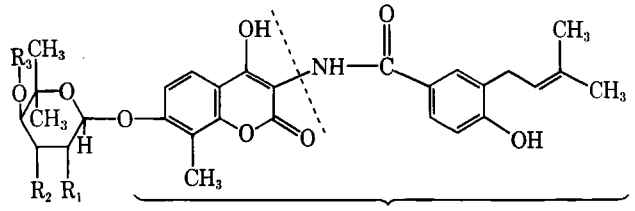
Abstract □ The effects of the use of ^{60}Co-irradiation to sterilize antibiotics were evaluated. The antibiotic powders were only occasionally contaminated with microorganisms. The *D*-values of the products and environmental isolates were 0.028, 0.027, 0.015, 0.046, 0.15, 0.018, and 0.19 Mrads for *Aspergillus* species (UC 7297, 7298), *A. fumigatus* (UC 7299), *Rhodotorula* species (UC 7300), *Penicillium oxalicum* (UC 7269), *Pseudomonas maltophilia* (UC 6855), and a biological indicator microorganism, *Bacillus pumilus* spores (ATCC 27142). An irradiation dose of 1.14 Mrads, therefore, was sufficient to achieve a six-log cycle destruction of *B. pumilus* spores. Based on the bioburden data, a minimum irradiation dose of 1.05 Mrads was calculated to be sufficient to obtain a 10^{-6} probability of sterilizing the most radioresistant isolate, *Pen. oxalicum*. To determine the radiolytic degradation scheme and the stability of the antibiotics following irradiation, high-performance liquid chromatographic (HPLC) methods were developed. The resulting rates of degradation for the antibiotics were 0.6, 1.2, 2.3, and 0.95%/Mrad for penicillin G, neomycin, novobiocin, and dihydrostreptomycin, respectively. Furthermore, radiolytic degradation pathways for the antibiotics were identified and found to be similar to those commonly encountered when antibiotics are subjected to acidic, basic, hydrolytic, or oxidative treatments. No radiolytic compounds unique to ^{60}Co-irradiation were found.

Keyphrases □ Irradiation—cobalt-60, alternate method for antibiotic sterilization □ Antibiotics—^{60}Co-irradiation as an alternate method for sterilization □ Sterilization—use of ^{60}Co-irradiation as an alternate method □ High-performance liquid chromatography—determination of radiolytic degradation scheme and stability of antibiotics following irradiation

The majority of pharmaceutical compounds are thermolabile. Sterilization of these compounds, therefore, is performed with the use of an ethylene oxide treatment. However, such a process has a low sterility assurance (1) and is quite costly. Moreover, the efficiency and safety of ethylene oxide as a sterilization agent are being questioned by both the Food and Drug Administration (FDA) (2) and the Environmental Protection Agency (3). ^{60}Co-Irradiation has been recognized as the preferred method for sterilization of medical devices and has been accepted for terminal sterilization of ophthalmic ointment (4). In many countries, products irradiated at 2.5 Mrads can be released for marketing as sterile without final sterility testing.

Recently the FDA proposed a guideline to regulate the irradiation of foods for human consumption (5). This proposal is based on a quantitative estimate of the projected daily human consumption of unique radiolytic compounds in irradiated foods (6). The daily dose of pharmaceutical products is substantially less than the amount of food intake. Thus, the feasibility of ^{60}Co-irradiation for the sterilization of antibiotics has been examined by assessing the resistance of product and environmental isolates to ^{60}Co-irradiation, determining stability, and identifying radiolytic compounds of ^{60}Co-treated antibiotics.



Novobiocic acid

Novenamine

	R_1	R_2	R_3
Novobiocin	OH	$OCONH_2$	CH_3
Isonovobiocin	$OCONH_2$	OH	CH_3
Descarbamylnovobiocin	OH	OH	CH_3
Desmethyldescarbamylnovobiocin	OH	OH	H
Dihydronovobiocin (reduction of isopent-2-enyl side chain to isopentyl)			

EXPERIMENTAL

^{60}Co-Irradiation—All irradiations were carried out[1], by raising the irradiation source, consisting of several rods containing ^{60}Co-pellets mounted on a plaque, from a pool of water into a stationary position for irradiation. Samples were placed in totes on two tiers surrounding the cobalt-60 source. A shuffle–dwell system averaged the radiation gradient around the source by periodically shifting the tote horizontally and vertically. Other samples were stationed at calibrated points where the radiation intensity was more precisely known.

Absorbed irradiation doses were measured potentiometrically using a ceric-cerous dosimeter[2] (7, 8) and spectrophotometrically using a red perspex dosimeter[3].

High-Performance Liquid Chromatography—The modular liquid chromatographic system consisted of a variable wavelength UV monitor[4] or a fixed wavelength UV monitor[5], a high pressure pump[6], a 20-μl fixed-loop injector[7], or a sample processor[8].

Chromatographic Conditions—*Penicillin G*—The chromatographic conditions were as described previously (9) using a reversed-phase column[9] with a mobile phase of acetonitrile, water, and 0.2 *M* ammonium acetate (50:40:10) at pH 4.0. The column effluent was monitored at 254 nm.

Neomycin—The chromatographic conditions were as described previously (10), which consisted of a precolumn derivatization of neomycin with 1-fluoro-2,4-dinitrobenzene at 100° for 40 min, followed by chromatography using a silica column[10] with a mobile phase of chloroform, tetrahydrofuran, methanol, and acetic acid (598:392:8:2). The column effluent was monitored at 254 nm.

[1] Conducted at a facility of Isomedix Inc., Morton Grove, Ill.
[2] Atomic Energy of Canada Limited, Ottawa, Canada.
[3] United Kingdom Atomic Energy Authority, Harwell, Oxon, U.K.
[4] Model 1201, Spectra Monitor I, Laboratory Data Control, Riviera Beach, Fla.
[5] 254 nm, model 1203, UV III Monitor, LDC.
[6] Milton Roy Mini Pump, LDC.
[7] Model 7010, Rheodyne, Berkeley, Calif.
[8] Intelligent Sample Processor (WISP), model 710B, Waters Associates, Milford, Mass.
[9] Partisil PXS 10/25 ODS, 10-μm particle size, or Partisil-10 ODS-3, 250 × 2.1-mm i.d., Whatman Inc., Clifton, N.J.
[10] SI-5A, Lichrosorb SI-100, 5-μm particle size, 250 × 4.6-mm i.d., Brownlee Labs, Santa Clara, Calif.

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Table I—Destruction of Microorganisms by ^{60}Co-Irradiation

Microorganism	*D*-Value, Mrads
Aspergillus species	
UC 7297	0.028
UC 7298	0.027
A. fumigatus (UC 7299)	0.015
Rhodotorula species (UC 7300)	0.046
Pen. oxalicum (UC 7269)	0.15
Pseud. maltophilia (UC 1885)	0.018
B. pumilus spore (ATCC 27142)	0.19

Novobiocin—The HPLC methods were as described previously (11). The normal-phase chromatography employed a silica column[10] with a mobile phase of 50% water-saturated butyl chloride, tetrahydrofuran, methanol, and acetic acid (88:5:4:3). A C_{18} column[11] was used for reversed-phase chromatography with 0.005 *M* 1-heptanesulfonate in methanol–water (80:20). The column effluent was monitored at 254 and 340 nm.

Dihydrostreptomycin—A reversed-phase RP-8 column[12] was maintained at 30°. A linear gradient elution program changed from mobile phase A to B in 30 min. Mobile phase A was 6% methanol; mobile phase B was 20% methanol, both contained hexanesulfonic acid, sodium sulfate, and acetic acid. The postcolumn oxidation and derivatization with hypochlorite and orthophthaldehyde (12) was applied for fluorometric detection of dihydrostreptomycin. A fluorometer[13] was used to monitor the column effluent.

Microbial Resistance to ^{60}Co-Irradiation—The resistance of microorganisms, isolated from the products and environment, to ^{60}Co-irradiation was investigated. The microorganisms examined were: *Aspergillus* species (UC 7297, 7298), *A. fumigatus* (UC 7299), *Rhodotorula* species (UC 7300), *Penicillium oxalicum* (UC 7269), and *Pseudomonas maltophilia* (UC 6888). Spores of *Bacillus pumilus* (ATCC 27142) were also used as biological indicator microorganisms.

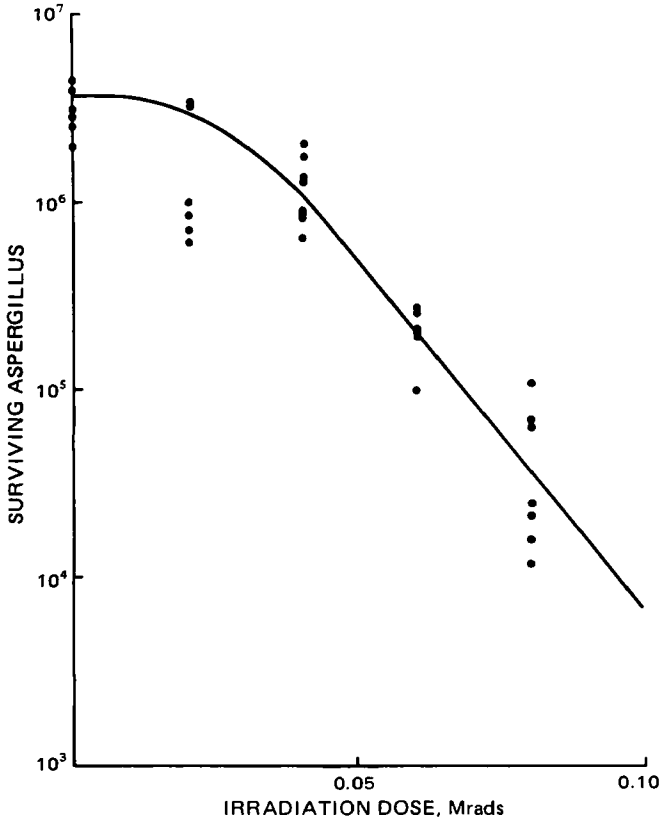


Figure 1—*Survival curve of* Aspergillus *species (UC 7298) when irradiated by cobalt-60 (D = 0.027).*

[11] Microparticulate Zorbax ODS, 250 × 4.6-mm i.d., DuPont Instrument, Wilmington, Del.

[12] RP-5A, 5-μm particle size, 250 × 4.6-mm i.d., Brownlee Labs, Santa Clara, Calif.

[13] Excitation at 360 nm, emission at 440 nm, Fluorichrom Varian, Palo Alto, Calif.

Table II—Stability of Neomycin Sulfate Powder to ^{60}Co-Irradiation

Irradiation Dose, Mrads	Neomycin B, μg/mg	Neomycin C, μg/mg
0	682.2	88.6
0.9	675.1	81.7
2.3	661.0	81.7
3.2	654.9	82.7
4.1	649.0	82.7
4.5	643.7	81.2

Table III—Stability of Penicillin G Procaine to ^{60}Co-Irradiation

Irradiation Dose, Mrads	Potency, Units
0	110.800
1.5	108.800
2.5	107.000
4.0	108.600

B. pumilus received doses of 0.0, 0.15, 0.3, 0.5, 0.7, and 0.93 Mrads, while all other microorganisms received 0, 0.02, 0.04, 0.06, and 0.08 Mrads of irradiation.

***D*-Value Determination**—Microbial resistance to ^{60}Co-irradiation was determined by the *D*-value, the irradiation dose in megarads required to achieve 90% reduction in the microbial population. The *D*-value was calculated using:

$$D = \frac{U}{\log a - \log b} \qquad \text{(Eq. 1)}$$

where *U* is the radiation in megarads, *a* is the initial microbial population prior to radiation treatment, and *b* is the microbial population surviving after treatment with *U* amount of radiation.

To determine the number of microorganisms surviving after receiving *U* amount of radiation, the samples were serially diluted, and 1.0 ml of the appropriate dilutions were plated in duplicate in trypticase soy agar[14].

RESULTS AND DISCUSSION

Microbial Resistance to ^{60}Co-Irradiation—Environmental and occasional microbial contaminants were isolated and used to examine resistance to ^{60}Co-irradiation. The survival curves of microorganisms irradiated followed first-order kinetics: when the log of survivors are plotted against the absorbed dose, a straight line is obtained. A typical

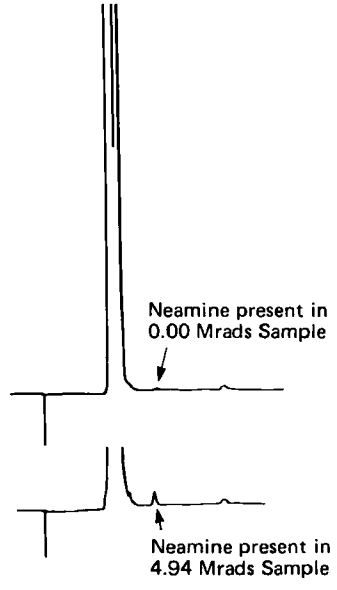


Figure 2—*HPLC indicating the increase in neamine when neomycin was irradiated by cobalt-60.*

[14] BBL, Division of Becton, Dickinson and Co., Cockeysville, Md.

Table IV—Effect of ^{60}Co-Irradiation on Stability of Sodium Novobiocin and Dihydrostreptomycin Sulfate Powder

Irradiation Dose, Mrads	Novobiocin, %	Dihydrostreptomycin, %
0	100	100
0.6	96.2	98.2
1.8	93.0	97.6
2.9	91.5	95.0
3.5	90.3	95.6
4.8	88.3	96.0
5.9	88.8	93.4

survival curve of an isolate, *Aspergillus* species (UC 7298), is shown in Fig. 1. Table I summarizes the *D*-values for *Aspergillus* species (UC 7297, 7298), *A. fumigatus* (UC 7299), *Rhodotorula* species (UC 7300), *Pen. oxalicum* (UC 7269), *Pseud. maltophilia* (UC 6888), and a biological indicator, *B. pumilus* (ATCC 27142) spores. The *D*-values of these microorganisms do not differ significantly from those values appearing in the literature (13–15).

Minimum Irradiation Dose (MID)—The highest *D*-value obtained in this study was 0.19 Mrads for the biological indicator microorganism, *B. pumilus*. Thus, 1.14 Mrads is sufficient to achieve a six-log cycle destruction of *B. pumilus* spores (0.19 × 6).

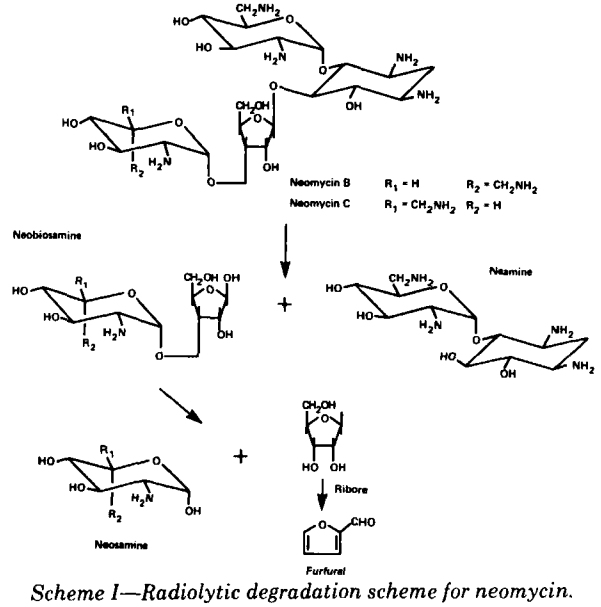
Bioburden data accumulated for 2 years indicated that <25% of a product contained >10 microorganisms/g of powder. The detection limit of the test is $\sim 10^1$ cells/g of sample. The MID required to achieve a 10^{-6} probability of sterility assurance was calculated by:

$$\text{MID} = D \times (6+1) \qquad \text{(Eq. 2)}$$

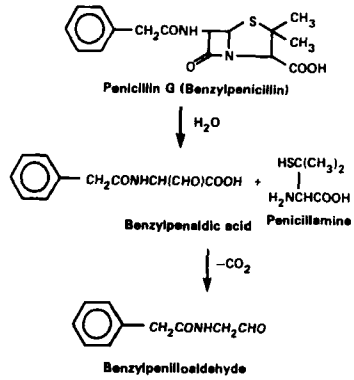
The most resistant isolate was *Pen. oxalicum* with a *D*-value of 0.15 Mrads (Table I). The MID for the product thus was calculated to be 1.05 Mrads. The 2.5-Mrads irradiation dose preferred by many countries for sterilization would result in an ~17-log cycle destruction of the most resistant isolate.

Stability of Antibiotics to ^{60}Co-Irradiation—To determine the feasibility of ^{60}Co-irradiation for the sterilization of antibiotics, stability to ^{60}Co-irradiation must be demonstrated and any unique radiolytic compounds identified.

The stability of neomycin sulfate, penicillin G procaine, sodium novobiocin, and dihydrostreptomycin following irradiation with cobalt 60 was examined by HPLC (Tables II–IV). The correlation between the HPLC and microbiological assay data was well established (9–11). The rates of degradation of penicillin G, neomycin, novobiocin, and dihydrostreptomycin by cobalt-60 were 0.6, 1.2, 2.3, and 0.95%/Mrad, respectively. If the ratio of maximum to minimum irradiation dose levels of a product is 1.3 (14), then the maximum irradiation dose that a product receives would be 1.37 Mrads (1.05 × 1.3), since the MID is 1.05. At 1.37 Mrads of irradiation, the expected percentages of degradation for penicillin G, neomycin, novobiocin, and dihydrostreptomycin are 0.8, 1.6, 3.2, and 1.3%, respectively. The rates of degradation for neomycins B and C were not statistically different (Table II).



Scheme I—Radiolytic degradation scheme for neomycin.



Scheme II—Radiolytic degradation scheme for penicillin G.

Identification of Radiolytic Degradation Compounds—Irradiated antibiotic powders were analyzed by stability-indicating HPLC to isolate, identify, and quantify radiolytic degradation compounds.

For positive identification, samples containing radiolytic compounds were extracted with solvents to enrich the compounds. Compounds were then purified using semipreparative scale HPLCs. The column effluents corresponding to the radiolytic compounds were collected and freeze dried. The identification of the radiolytic compounds was confirmed by a comparison of their mass spectra with those of authentic samples.

Neomycin—A nonirradiated sample and one irradiated at 5.0 Mrads were analyzed by HPLC. The nonirradiated sample contained 39.3 mg of total neomycin (neomycins B and C)/g of a product, while the 5.0-Mrads irradiated sample contained 34.8 mg/g: a decrease of 4.5 mg of neomycin/g. An inspection of HPLC chromatograms indicated an increase in the neamine peak (Fig. 2). The nonirradiated sample contained 0.8 mg of neamine/g of the product, while the irradiated sample contained 3.3 mg/g: a difference of 2.5 mg of neamine/g. A degradation of 4.5 mg of

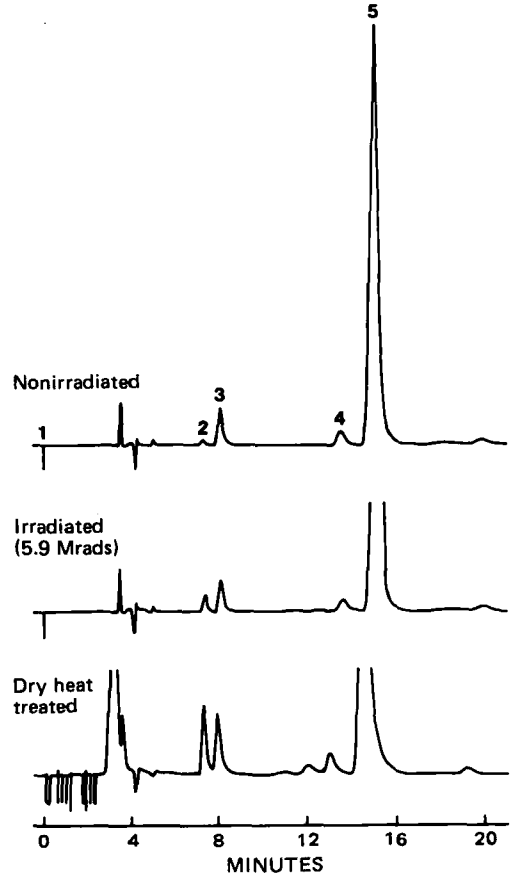


Figure 3—*Normal phase HPLC of novobiocin. Key: (1) injection; (2) radiolytic compound; (3) descarbamyl novobiocin; (4) isonovobiocin; (5) novobiocin.*

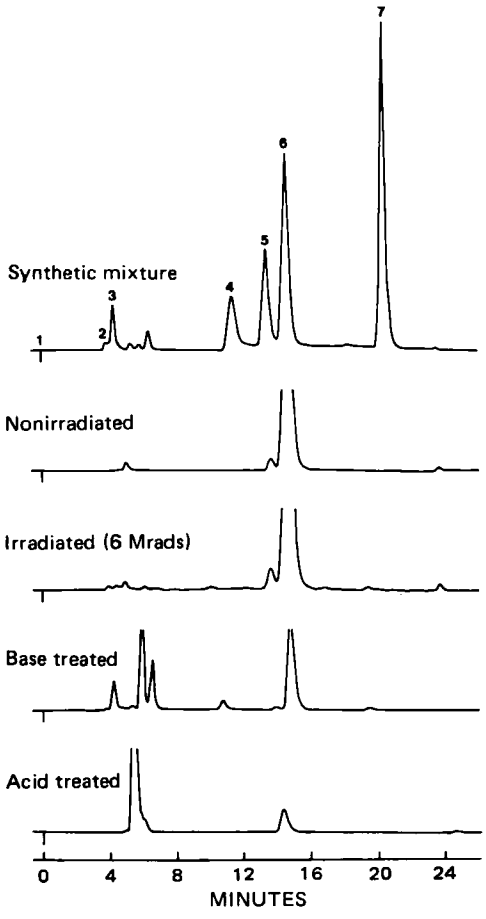


Figure 4—*HPLC of dihydrostreptomycin treated with irradiation, base, and acid. Peak identification: (1) injection; (2) streptamine; (3) D-glucosamine; (4) streptomycin; (5) dideguanyldihydrostreptomycin; (6) dihydrostreptomycin; (7) β-methyldihydrostreptobiosaminide.*

neomycin theoretically yields 2.4 mg of neamine. This calculated quantity of neamine is not statistically different from the observed value of 2.5 mg. Furthermore, a peak corresponding to neosamine increased, but no significant increase of the neobiosamine peak was observed. Thus, it may be concluded that neomycin, upon irradiation, undergoes hydrolytic cleavage at a glycosidic bond to form neamine and neobiosamine. Neobiosamine further degrades to neosamine and ribose; the latter likely yields furfural. A postulated radiolytic degradation scheme is shown in Scheme I. This is the common, hydrolytic degradation pathway for neomycin (16).

Penicillin G—The radiolytic degradation scheme for penicillin G was similarly identified and is shown in Scheme II (9). Penicillin G underwent hydrolytic cleavage to form benzylpenillic acid and benzylpenicilaldehyde. These degradation compounds may also be formed through alkaline treatment (17). Benzylpenicilloic acid, benzylpenilloic acid, and hydroxybenzylpenicillin, reported to form when penicillin G is irradiated in an aqueous solution (18), were not detected.

Novobiocin—Upon examination of the HPLC chromatogram of irradiated and nonirradiated novobiocin powder (Fig. 3), it became evident that one peak, which was originally present in the bulk powder, increased upon irradiation. This degradation compound can easily form upon dry heat treatment of novobiocin powder (Fig. 3). No new peak unique to ^{60}Co-irradiation was detected. Relative chromatographic retention of this peak (0.50) is close to but not identical to that of novobiocic acid (0.44); moreover this peak lacks absorptivity at 340 nm. Novobiocic acid strongly absorbs at 340 nm. The column fraction corresponding to this unknown peak was collected and analyzed by mass spectrometry. The mass spectrometric data clearly identified the unknown compound as the ring A amide (11). The site of the radiolytic cleavage of the novobiocin molecule is shown in the structure of novobiocin.

Dihydrostreptomycin—Identification of degradation compounds and the radiolytic pathway for dihydrostreptomycin are in progress. However, as shown in Fig. 4, the compounds that increase by irradiation were either inherently present in commercially available, nonirradiated lots or can easily be formed by acidic or basic treatment. No new compounds peculiar to irradiation were found.

^{60}Co-Irradiation has been shown to be an effective means of sterilizing antibiotics. The minimum irradiation dose required to achieve 10^{-6} probability of sterilizing the most resistant isolate, *Pen. oxalicum*, is 1.05 Mrads, degradation of antibiotics from ^{60}Co-irradiation is minimal, and the radiolytic degradation schemes are similar to those commonly encountered when antibiotics are subjected to acidic, basic, hydrolytic, or oxidative treatment. No radiolytic compounds unique to irradiation have been found.

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