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RADIOSTERILIZATION OF SULFONAMIDES II: DETERMINATION OF THE EFFECTS OF GAMMA IRRADIATION ON COMMERCIAL SULFONAMIDE PREPARATIONS

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Abstract

In the present study, the effects of different doses of gamma radiation on two different members of sulfonamide (sulfacetamide and sulfametoxazole) commercial preparation were studied for the evaluation of the physibility of radiation as a sterilization method for this group of antibacterial agent. HPLC technique was performed to determine the radiolytic intermediates. All investigations were done in accordance with the requiring pharmacopoeia specification for pharmaceutical dosage forms. Because of being antibacterial in nature antimicrobial activity loss was also investigated in irradiated samples. Studies performed under normal environmental conditions were repeated for samples stored under accelerated stability conditions to investigate possible degradation mechanism and kinetics of irradiated dosage forms. Sulfanilamide was determined as degradation product by HPLC method. Both because of the low quantity and this group of drugs' showing their pharmacological effects by this metabolite, irradiated samples were considered chemically stable. No microbial activity loss and no harmful effect resulting from degradation products were seen in biological investigations also. As a result these commercial preparations were found to be stable in studied radiation doses. This study could be a model for industrial physibility studies of the radiosterilization of sulfa group drugs.

Keywords: *Gamma irradiation; Sterilization;Rradiation sterilization; Sulfonamides,*

Sulfonamidlerin Radyasyonla Sterilizasyonu II: Gamma Radyasyonun Ticari Sulfonamit Preparatları Üzerine Etkilerinin Değerlendirilmesi

Bu çalışmada konvansiyonel sterilizasyon tekniklerine alternatif olarak gamma radyasyonun etkileri sulfonamit grubu iki etkin madde (sulfametoksazol ve sulfasetamit) içeren piyasa preparatları üzerinde araştırılmıştır. Radyolitik ara ürünler yüksek basınçlı sıvı kromotografisi (HPLC) ile belirlemiştir. Işınlama sonrası farmasötik dozaj formları farmakope uygunlukları bakımından değerlendirilmiştir.Bunun yanısıra radyasyona tabii tutulan numunelerde antimikrobiyal aktivite kaybı olup olmadığı da incelenmiştir.Işınlanan numunelerin raf ömürlerinin belirlenmesi için farklı saklama koşullarındaki stabiliteleri de üç aylık periyotlarda incelenmiştir.Işınlama sonrası sulfonamit bozunma ürünü olarak saptanmış olsa da gerek miktarının çok düşük olması gerekse sülfa grubu ajanların antimikrobiyal etkilerinden bu metabolitin sorumlu olması nedeni ile çalışılan radyasyon dozlarında numuneler kimyasal olarak stabil bulunmuştur. Mikrobiyal aktivite kaybının olmaması da bu sonucu desteklemektedir.Bu çalışma sülfa grubu ilaçların endüstriyel olarak radyasyonla sterilizasyonu için model teşkil etmektedir.

Anahtar Kelimeler: *Gama ışınlaması, Sterilizasyon, Radyasyon ile sterilizasyon, Sulfonamidler*

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INTRODUCTION

Although every day new antibacterial agent takes place in the pharmaceutical market; sulfonamide group of antibacterial agents still have important place in this market with having a broad spectrum of use in bacterial infections. Sulfacetamide sodium and sulfamethoxazole are two important members of this group.

In the pharmaceutical industry; sterilization process is a very important step for pharmaceutical dosage forms that are administrated to humans by both parenterally and ophthalmologically. For the sterilization of these pharmaceutical dosage forms, radiation sterilization is an alternative method (1-4).However, radiation sterilization has many advantages to other sterilization techniques like no thermal increase during the sterilization process or no toxic residue (especially occurring with ethylene oxide sterilization) and high penetration capacity; chemical mechanisms induced by radiolysis (especially in aqueous systems) is the major disadvantage of the radiation process. Therefore, radiation sterilized pharmaceutical dosage forms must be examined whether degradation products are formed, as if; it has to be determined that they are in small quantities (Pharmacopeias < 0.19 %) and not harmful. Together with the chemical stability, prevention of pharmacological activity and dosage form integrity of the irradiated preparation must be confirmed.

Our previous investigation on the sulfanilamide group of powders (sulfacetamide sodium (SSA-Na) and sulfamethoxasole (SMZ) (5) let us to evaluate the effects of gamma irradiation on their pharmaceutical dosage forms: ophthalmic suspensions, ophthalmic solutions and parenteral solutions, both chemically, microbiologically and biologically.

MATERIALS and METHODS

Dosage forms were provided from İbrahim Ethem Corp. (Turkey), Abdi İbrahim Corp. (Turkey), Roche Corp. (Turkey) and Glaxo Smith Kline Corp. (Turkey). Pharmaceutical dosage forms and their specifications are listed below:

Code	***Active Substance***	***Dosage Form***
A	SSA-Na (100mg)	Ophthalmic suspension
B	SSA-Na (500 mg)	Ophthalmic suspension
C	SMZ (500 mg)	i.v infusion solution
D	SMZ (100 mg)	i.m solution

Irradiation Process

Dosage forms in their final package forms were irradiated at the doses of 5, 10, 25 and 50 kGy. All irradiations were performed at room temperature (~20 °C) using a ^{60}Co Gamma Cell 220 as ionizing radiation, source of dose rate 2.84 kGy.h^{-1}. The actual doses received by samples were determined by measuring the change in the absorbance of red perspex dosimeters (Harwell 4034) at 603 nm attached to the sample package during the irradiation period. The corresponding doses were obtained from a calibrating graph. Administered doses were accurate ±3% (at 95% confidence level).

Studies carried on samples under normal conditions

Unirradiated samples were used as controls to detect physicochemical, chemical, biological and antimicrobial activity changes resulting from the action of ionizing radiation.

Physicochemical properties

While irradiated ophthalmic suspensions were evaluated for appearance, pH changes, sedimentation and resuspendability, particle size and size distribution, density and viscosity; irradiated parenteral solutions were evaluated for appearance, pH changes, density, homogenity and particular material.

Determination of degradation products

Determination of radicals produced in irradiated samples by ESR technique failed due to short life of these radicals. Therefore HPLC technique was performed to determine the radiolysis products. In this procedure two different HPLC methods were used to determine the degradation products of formulations (6-7). Following chromatographic conditions were applied.

SSA-Na	
Column	Phenomenex L1 (C18) (25 cm x 4.6 mm) (5μm)
Mobile phase	Water-methanol-glacial acetic acid (89:10:1) (pH:2.5)
Flow rate	1.5 ml. min^{-1}
Injection volume	30 μl
Detection	UV Detector (254 nm)
Standard	0.3 mg/ml in methanol
solution	0.15 mg/ml in methanol
Sample solution	

SMZ	
Column	Waters Spherisorb S5W (250 mm x 4 mm) (10μm)
Mobile phase	Cyclohexane-ethanol-glacial acetic acid (85.7: 11.4: 2)
Flow rate	2 ml. min^{-1}
Injection volume	4 μl
Detection	UV Detector (260 nm)
Standard	0.5 mg/ml in chloroform-methanol (1:1)
solution	0.5 mg/ml in chloroform-methanol (1:1)
Sample solution	

Antimicrobial Activity

Antibacterial activities of irradiated and unirradiated preparations were performed by the microdilution method recommended by National Committee for Clinical Laboratory Standards (8). According to this procedure microorganism inoculum was prepared first then antimicrobial activity was determined against these reference microorganisms: Staphylococcus aureus (S.aureus) (ATCC 25923), Escherichia coli (E.coli) (ATCC 25922), Enterococcus faecalis (E. faecalis) (ATCC 29212), Pseudomonas aeruginosa (Ps. aeruginosa) (ATCC 27853). The results were expressed as minimum inhibitory concentrations (MIC).

Preparation of microorganism inoculum

Before the test each microorganisms were incubated in Muller –Hinton broth for 2-5 hours at 35 °C. Microorganism concentration was adjusted to 0.5 McFarland-standard (0.5-1 x 10^8 cfu. ml^{-1}). Microorganism suspension was diluted to be 5.5 x 10^5 cfu. ml^{-1} in the well.

Microdilution broth method

In the test, 96 well, micro-titer trays were used. Two fold dilutions of irradiated and unirradiated preparations were prepared in Muller-Hinton broth in the well of the plates. Each sample was diluted from 1-11 wells of the micro-titer trays (1/4 to 1/4096 dilutions). Previously prepared microorganism suspensions were added to each well and the plates were incubated 18-24 hours at 35 °C. Minimum inhibitory concentrations (MIC, $\mu g.ml^{-1}$) were defined as the lowest concentrations (dilution) of the samples that inhibited visible growth of the microorganism.

Biologic Evaluation

While ophthalmic preparations were evaluated for irritation; parenteral solutions were evaluated for toxicity. Ethic committee acceptance was obtained for biological studies.

Eye Irritation

For the evaluation of irritation in the animal eyes of irradiated ophthalmic preparations modified Draize test was used (10). In this procedure 6 female Albino rabbits were used. The right eyes of the animals were used as control and treated with unirradiated preparations. 30 µl of irradiated samples were instilled into the left eye of the rabbits and animals were examined for chemosis, iritis and conjunctival hyperemia during the 7 days period by the ophthalmologist.

Minimum Lethal Dose (LD_{50}) Determination and Acute Toxicity Studies

Because of the fact that minimum lethal dose of SMZ for both i.v and i.m administration routes could not be found; LD_{50} was determined for both i.v and i.m routes before the toxicity studies of irradiated samples. For LD_{50} determination study, Approximate Lethal Dose Method (Deichman) was used (11). In this procedure an arbitrary dose was applied to the first group of animal containing 6 male mice. After surviving the first group of animals, increasing doses were administered until lethal dose was achieved. Values were calculated by the graphs that compare statistically probit values of mortality versus administration dose.

Toxicity study was performed by Acute Toxicity Study (12). In this process, radiation sterilized parenteral solutions were administrated by both i.v and i.m route respectively to the five mice of each sex at a determined LD_{50} dose previously. After administration of the irradiated drugs, animals were evaluated for criteria of toxic effects: mortality, increase in body weight, food consumption during 7 days period.

Studies carried out under accelerated conditions

In this part of the work, studies performed under normal environmental conditions (except HPLC evaluation) were repeated for samples stored in a climate chamber in their final package forms under accelerated stability conditions like high temperature (40±2)°C and high relative humidity (75±5)% conditions over a period of three months to investigate possible degradation mechanism and kinetics of irradiated dosage forms. Samples were stored in the chamber continuously and aliquots were taken of for measurements at room temperature. Unirradiated samples were used as negative controls for comparison and measurements were repeated every week of the first month and then on monthly basis.

RESULTS

Studies carried out under normal conditions

Physicochemical properties

Color and pH change of ophthalmic dosage forms were observed with increasing applied radiation dose. The results obtained for pH values were given in Table 1. As seen from these data, with in experimental error irradiated samples have the same pH values. However, these values are well above of that calculated for control. Parenteral solutions were also studied from homogeneity and particular matter point of view and it was concluded that homogeneity stays constant in the applied dose range (5-50 kGy).However, average particle size of 50 kGy irradiated suspensions increased significantly; no sedimentation was observed.

Table 1. pH values of irradiated samples

Code	Dosage Form	Active Substance	pH					
			Reference Values	Applied Dose (kGy)				
				Control	5	10	25	50
A	Opht. Susp	SSA-Na	6.00-7.40	6.75±0.01	7.03±0.02	7.04±0.04	7.06±0.01	7.06±0.02
B	Opht. Susp	SSA-Na	6.00-7.40	6.90±0.01	7.23±0.01	7.23±0.04	7.24±0.04	7.28±0.02
C	Parent. Sol	SMZ	9.50-10.50	9.76±0.02	10.13±0.02	10.15±0.02	10.09±0.02	10.07±0.02
D	Parent. Sol	SMZ	9.50-10.50	10.20±0.02	11.08±0.04	11.09±0.03	11.03±0.04	11.84±0.06

Determination of Degradation Products

In the HPLC chromatograms of irradiated ophthalmic suspensions three peaks were observed. However, one of them was identified as sulfanilamide; other two peaks could not be identified. HPLC chromatograms of irradiated and unirradiated (control) suspensions were given in Figure 1. The amount of degradation product (sulfanilamide) increased with the increase in radiation dose; it was in small quantities (G (sulfanilamide): $0.043.10^{-7}$ and $0.055.10^{-7}$ mole.joule^{-1} for two different formulations).

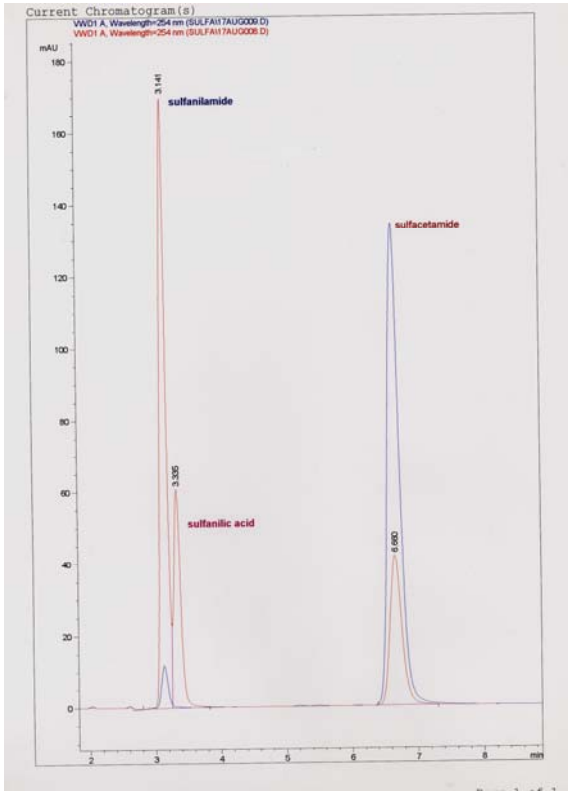

Fig 1.a HPLC chromatograms of reference samples and 5 kGy irradiated SSA-Na ophthalmic suspension

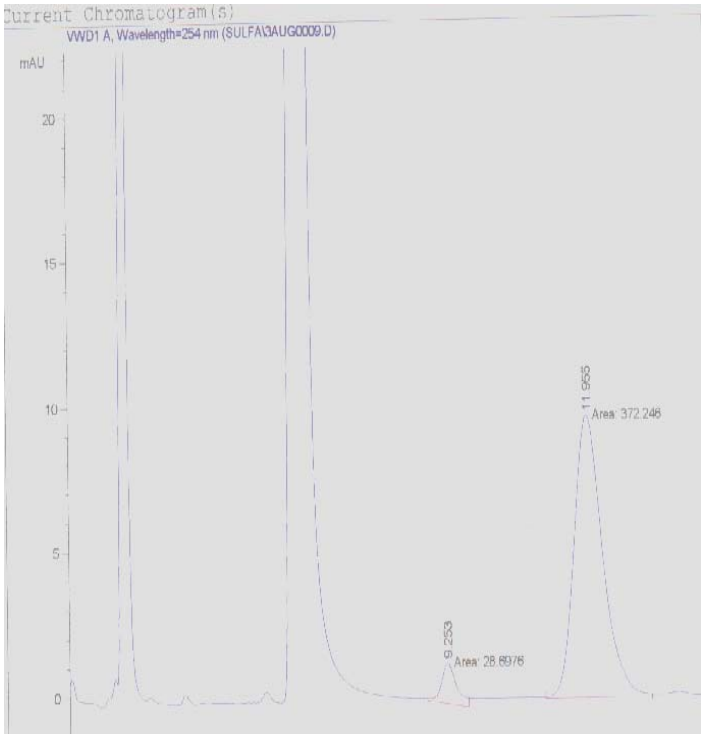

Fig. 1.b Unidentified peaks of 5 kGy irradiated ophthalmic suspension

In parenteral solutions no degradation product was observed with the irradiation of these formulations and active substances' (SMZ) amount did not change with the irradiation also (Figure 2).

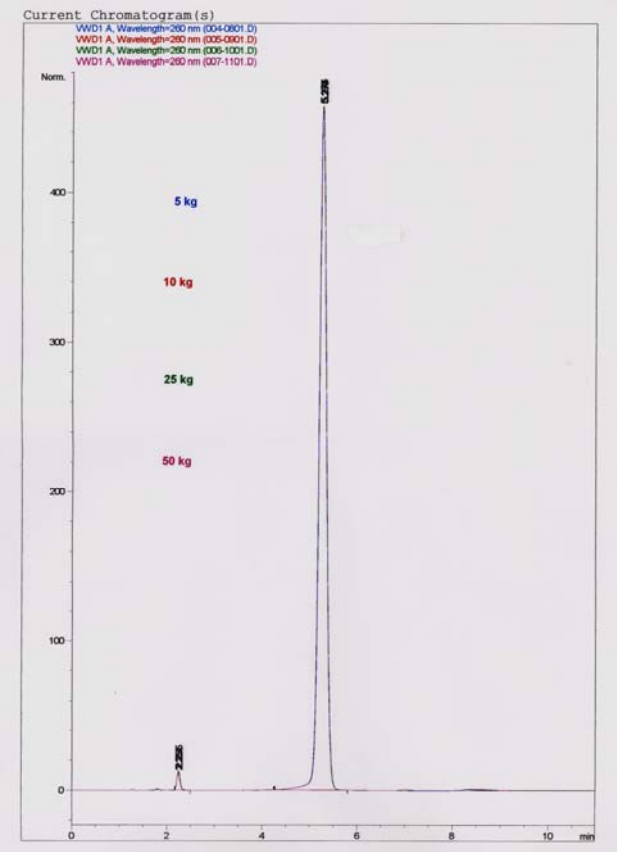

Figure 2. HPLC chromatograms of irradiated SMZ parenteral solutions

Microbiologic Evaluations

MIC values were determined by microdilution method using *E. coli, E. faecalis* and *Ps. aeruginosa* strains. Slight decreases compared to unirradiated (control) sample in MIC values for irradiated dosage forms were observed as seen Table 2.

Table 2. MIC values of irradiated samples

Dosage Form	Bacteria	MIC (μg.ml^{-1})				
		Control	Applied Dose (kGy)			
			5	10	25	50
A	**E. coli**	1.22	1.22	1.22	1.22	1.22
	E. faecalis	1.22	1.22	1.22	2.44	2.44
	Ps. aeruginosa	78.12	78.12	78.12	78.12	78.12
B	**E. coli**	48.85	48.85	48.85	48.85	48.85
	E. faecalis	195.30	195.30	195.30	195.30	195.30
	Ps. aeruginosa	195.30	195.30	195.30	390.06	781.25
C	**E. coli**	6.10	6.10	6.10	6.10	6.10
	E. faecalis	6.10	6.10	6.10	6.10	6.10
	Ps. aeruginosa	6.10	6.10	6.10	6.10	6.10
D	**E. coli**	1.22	1.22	1.22	1.22	1.22
	E. faecalis	1.22	1.22	1.22	1.22	1.22
	Ps. aeruginosa	1.22	1.22	1.22	1.22	1.22

Biologic Evaluations

In the irritation study of irradiated ophthalmic suspensions; 30 female albino rabbits, that is six animals per irradiation dose, were used throughout the experiments. Nothing was applied to the left eye of the animals, namely they are considered as controls, while to the right eye unirradiated and irradiated ophthalmic dosage forms were applied. The state of the eyes of the animals before and after application of ophthalmic suspension were studied, each day by the ophthalmologist; although no irritation was observed in animal eyes treated irradiated dosage forms, in the higher irradiation dose (50 kGy) redness in animal eyes was observed. Irritation test results were illustrated in Table 3.

Table 3. Eye irritation test results of irradiated ophthalmic preparations

Dosage Form	Examination Area	Positive Results / Total Animal			
		5 kGy	10 kGy	25 kGy	50 kGy
A	Cornea	0/6	0/6	0/6	0/6
	Conjunctiva	16	1/6	1/6	1/6
	Iris	0/6	0/6	0/6	0/6
B	Cornea	0/6	0/6	0/6	0/6
	Conjunctiva	1/6	1/6	1/6	2/6
	Iris	0/6	0/6	0/6	0/6

For the evaluation of irradiated parenteral preparations after determination of LD_{50} values of the active substance (SMZ) for both i.v and i.m route (1840 mg.kg^{-1} and 2442 mg.kg^{-1} respectively);

acute toxicity test was carried out. In this test although no toxic effect was found for parenteral solutions when compared with the control group (unirradiated), significant physical changes were observed in animals that treated high radiation dose (50 kGy). The results were illustrated in Table 4.

Table 4. Acute toxicity test results of parenteral solutions

Dosage Form and Administration Route	Determined LD_{50}	Dose & Sex	Mortality	Observed Changes in Animals
D (i.v)	1840	**Control(Unirradiated)**		
		Male	1/5	-
		Female	2/5	-
		Total	3/10	
		5kGy		
		Male	2/5	-
		Female	2/5	-
		Total	4/10	
		10kGy		
		Male	2/5	-
		Female	2/5	-
		Total	4/10	
		25kGy		
		Male	2/5	-
		Female	1/5	-
		Total	3/10	
		50kGy		
		Male	1/5	Loss of fur in one mouse
		Female	3/5	Loss of fur in two mice
		Total	4/10	
E (i.m)	2442	**Control (unirradiated)**		
		Male	2/5	-
		Female	2/5	-
		Total	4/10	
		5kGy		
		Male	3/5	-
		Female	1/5	-
		Total	4/10	
		10kGy		
		Male	1/5	Opacity in one mouse's eye
		Female	2/5	-
		Total	3/10	
		25kGy		
		Male	2/5	-
		Female	2/5	-
		Total	4/10	
		50kGy		
		Male	1/5	-
		Female	3/5	Splitting tail in one mouse
		Total	4/10	-

Studies carried out under accelerated conditions

Conservation of color change brought about by irradiation of commercial preparations, throughout the stability test experiments point out the stability of intact sulfonamide molecules and radiolytic intermediates produced by radiation in the commercial preparations even under accelerated stability conditions. A meaningful increase in pH values of control samples but not in irradiated samples with increase in storage time indicates that stability conditions might produce the same effect on intact sulfanilamide molecules as radiation produce. In other words, at high temperature and high relative humidity sulfonamide molecules can undergo degradation. The attempt to detect the radical or radical accompanying this degradation in preparations under severe stability test conditions was unsuccessful as in the case of irradiated preparations due to the short life of the generated radicals.

DISCUSSION

Gamma radiation transfers its energy rather indirectly to target molecules in solutions. Radicals produced by the direct action of radiation on water molecules are the principal elements in the degradation of aqueous solutions. In its direct action, gamma radiation ejects electrons from water molecules. Positively charged water molecules react in their turn, react with uncharged water molecules and many radicals, mainly OH· are produced which are very strong oxidants and play principal role in the degradation of aqueous systems. This feature of water molecule makes the aqueous systems more sensitive to radiolysis.

Irradiation with gamma radiation of commercial sulfacetamide ophthalmic suspensions is found to cause a color change from yellow to amber which increases with the increase in the applied dose. Sulfanilamide detected by HPLC technique among three other radiolytic intermediates is believed to be at the origin of color changes (13). Sulfanilamide has also been detected in sulfacetamide solutions irradiated in oxygen (14). The increase in color change with the increase in the radiation dose thus the increase in sulfanilamide was considered as the justification of the responsibility of sulfanilamide from the color change. Although sulfanilamide seems an undesirable radiolytic product causing color change in the preparation, it is basic molecule of sulfonamides group having direct pharmaceutical effect. As is known, sulfonamides exert their pharmacological effect through this metabolite. In other word, although discoloration due to irradiation might seem an undesirable result for the drug, it has no harmful effect when this drug is applied to the human organism.

Investigations carried out on 10^{-4}-1.2 M solute concentrations of SSA-Na by Philips et al (14) have shown that both the major species of water radiolysis, e^-_{aq} and OH• radicals participate in the degradation of SSA-Na. Extremely high reactivity of sulfacetamide toward e^-_{aq} and the very significant participation of OH• radical have been demonstrated by these authors, and four products of irradiation, sulfanilic acid, hydroxylated sulfacetamide and two unidenfied, were observed. However, the solute concentrations have been observed to influence the disappearance of sulfacetamide. In concentrated solutions and suspensions normally used in eye preparations (20% and 30%), the decomposition by a dose as large as 50 kGy have been found to be not greater than 4% which agrees well with the results obtained in the present work relative to SSA-Na ophthalmic suspensions. In 1.2 M SSA-Na solutions, no direct action contributions would be expected due to the stability of sulfacetamide to γ-irradiation in solid state (14). This result is extremely satisfactory for radiosterilization of concentrated sulfonamide solutions and/or suspensions compared with alternative conventional sterilization procedures. Sulfanilic acid determined as degradation product in dilute sulfacetamide solutions irradiated at a dose of 25 kGy in triple distilled water by Philips et al (15) was not observed in the present work. The absence of sulfanilic acid among the radiolytic among the radiolytic product might be

due to different buffer capacity of mobile phase used in the present work or different formulation of active substance.

Antimicrobial activity results reported in the present study for preparations are consistent with the results of Trigger and Caldwell (16) who have reported the same activity results for the ophthalmic solutions and ophthalmic suspensions of sulfacetamide sodium (SSA-Na) irradiated at 25 kGy. Experimental results show that the decrease in antimicrobial activity with increase in applied radiation dose for solid sulfonamides is higher than the antimicrobial activity of commercial preparations containing same active component. This difference in antimicrobial activity might be due to the preservative substances added during the production of the preparations. In general, sulfa drugs of SMZ and SSA-Na have been observed to keep their antibacterial activity before and after irradiation irrelevant from irradiation dose level and host.

Irritation test results, on rabbits carried out using commercial ophthalmic preparations irradiated up to 50 kGy have shown that not creating any irritation on the eyes of animals except some conjuntivity and a characteristic sensitivity increase probably due to the H_2O_2 at high radiation dose (50 kGy). Similarly in the toxicity study, however, no toxic effect was observed in animals; except some physical changes in higher radiation dose (50 kGy).

As a result; although the radiolytic intermediates couldn't be determined due to their rather short half life, when the overall evaluation of all tests are taken into consideration it can be concluded that OH^-, H_3O^+, and H_2O_2 are produced as radiolytic intermediates in pharmaceutical dosage forms. As previously mentioned, sulfanilamide was determined as degradation product by HPLC method. Both because of the low quantity of sulfanilamide formed as a degradation product and this group of drugs' showing their pharmacological effects by this metabolite, and also because of observing no harmful effect resulting from degradation products in biological investigations show that in the studied commercial preparations irradiation cause no problem other than discoloration. A similar discoloration problem was also observed during thermal sterilization of this group of drugs (13). This discoloration could be prevented either by adding appropriate preservatives into the formulations or by changing the process conditions of irradiation (lowering the temperature, absence of oxygen). Also in the study with the packaging materials, no change in pharmacopoeia specifications (color, heavy metals and nonvolatile residue, pH, UV detection) of the plastic packaging materials in applied radiation doses was observed (unpublished data). However no degradation product was determined in the higher radiation doses, lower radiation doses are industrially promising both for cost-effective and for lower radiation risk for the radiosterilization of this group of drugs. This study could be a model for industrial physibility studies of the radiosterilization of sulfa group drugs.

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