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RESEARCH ARTICLE

The Effects of Gamma Radiation Sterilization on Cefazolin Sodium

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The Effects of Gamma Radiation Sterilization on Cefazolin Sodium
Summary

An antibiotic, cefazolin sodium (CFZ-Na), belonging to cephalosporins was irradiated and the changes in physico-chemical properties, pH, melting point, UV, IR, NMR, TLC, HPLC, ESR and antimicrobial activities of active components were studied at normal and accelerated stability test conditions. The color of CFZ-Na powder changed from white to yellow upon irradiation. The change in the color was kept throughout the irradiation process. Two radiolytic intermediates for CFZ-Na were found in the irradiated solid samples after ESR analysis. The results obtained under normal and accelerated stability test conditions were observed to be consistent with the control values.
Key Words: *sterilization, gamma irradiation, ESR, cefazolin sodium, stability.*
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Gama Radyasyonuyla Sterilizasyonun Sefazolin Sodyum Üzerine Etkileri
Özet

Sefalosporin grubu bir antibiyotik olan Sefazolin Sodyum (CFZ-Na) gama radyasyonu ile ışınlandı. Işınlama sonrasında, normal ve hızlandırılmış stabilite koşullarında etkin maddenin fizikokimyasal özellikleri, pH, erime derecesi, UV, IR, NMR, TLC, HPLC, ESR ve antimikrobiyal aktivitesi değerlendirildi. Yapılan çalışmalar sonucunda, toz CFZ-Na'un renginin ışınlama etkisi ile beyazdan sarıya değiştiği gözlenmiştir. Ayrıca ESR analizleri ile ışınlama sonrası iki radyolitik ürün tespit edilmiştir.
Anahtar Kelimeler : *Sterilizasyon, gama ışınlama, ESR, sefazolin sodyum, stabilite.*

INTRODUCTION

Conventional techniques currently used for sterilization of pharmaceuticals have some disadvantages, such as heat, humidity, long quarantine period and inappropriate dosage forms. The gamma irradiation technique has several advantages, including having no residuals and no significant temperature changes, so it can be applied on heat-sensitive products. In addition, it has a relatively easy validation procedure (like dose mapping, identification quantitation and safety assessments of the impurities (free radicals) generated) and the only necessity to achieve sterilization is control of the dose required to the sample. Therefore, the use of gamma rays for the sterilization of pharmaceutical raw materials and dosage forms is an alternative method for sterilization[1-3]. However, one of the major problems of radiosterilization is the production of new radiolytic products during the irradiation process[4]. For that reason, the main problem in radiosterilization is to determine and to evaluate these physical and chemical changes originating from high-energy radiation[5-8].

The aim of this study was to investigate the nature and extent of radio-induced changes in physico-chemical and microbiological properties of cefazolin sodium (CFZ-Na) after irradiation in a dose range of

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5-40 kGy. Various sensitive techniques such as spectroscopic, chromatographic and microbiological were used as well as pH, melting point, and solubility measurements in order to evaluate any changes. The results of control and irradiated CFZ-Na powder under normal and accelerated test conditions are comparatively presented.

MATERIALS and METHODS

CFZ-Na powder was kindly provided from Mustafa Nevzat Corp. and I.E. Ulagay (Turkey). Solid samples were used without any purification in powder form. The following procedure was adopted for the irradiation of solid CFZ-Na samples. The other ingredients were of analytical grade.
Normal and accelerated types were applied as the stability test conditions. 25˚C and 40˚C and 60% and 75% relative humidity were parameters of climate chamber for normal and accelerated stability tests, respectively, throughout three months, depending on stability regulations applied by the Turkish Ministry of Health[9, 10].
Student's t-test was used as for statistical evaluations.

1. Irradiation Process

Irradiation was performed at room temperature using a ^{60}Co (Gamma 220) source available at the Turkish Atomic Energy Agency Facility (TAEK) at a dose rate of 1.88 kGy.h-1. Solid samples in Type I glass vials were irradiated at the doses of 5, 10, 25 and 40 kGy.

2. Studies Carried out under Normal Conditions

Control samples were used to detect physico-chemical and antimicrobial activity changes arising from the effect of ionizing radiation on CFZ-Na. 25˚C temperature and 60% relative humidity were accepted as normal test condition. Studies were carried out for three months.

2.1. Physico-Chemical Properties

To determine physico-chemical changes, tests with respect to organoleptic properties, pH, solubility and melting point were performed. In these studies, pH meter (WTW Inolab, Germany), stirrer (Heidolph-RZR1, USA) and melting-point apparatus (Thomas Hoover Capillary Melting Point Apparatus, USA) were used, respectively. The solvents were at the purity of analytical grade. The obtained results were compared with the pharmacopeial requirements[11].

pH measurements of the control (non-irradiated) and irradiated CFZ-Na were performed using 10 $mg.mL^{-1}$ aqueous solution of these samples. Solubility changes of 1 g CFZ-Na in different solvents such as water, acetone, methanol and chloroform and changes in melting point were also investigated. For organoleptic properties appearance, color and crystallinity were evaluated before and after the irradiation.

2.2. Spectroscopic Methods

Changes in spectral properties of control and irradiated solid samples were studied with Infra Red (IR), Ultraviolet (UV), Nuclear Magnetic Resonance (NMR) and Electron Spin Resonance (ESR) techniques. IR (Bruker, Germany) spectra were recorded for control and irradiated powders in KBr matrix. λ_{max} values in distilled water were determined for UV (Shimadzu UV 160 A, Japan) analysis.

NMR analysis was performed using proton-NMR spectrometer (Bruker GmbH DPX-400, 400 MHz, Digital FT-NMR, Germany) on control and irradiated samples dissolved in DMSO. Tetramethylsilane was used as the internal standard.

ESR measurements were carried out using a Varian 9≤-E line-X band ESR spectrometer (USA) operating at 9.5 GHz and equipped with a TE104 rectangular double cavity containing a standard sample (strong pitch) in the rear resonator, which remained untouched throughout the experiment. Spectrometer settings were adopted throughout the experiments as: central field 327.5 mT; sweep width 10 mT; microwave frequency 9.2 GHz; microwave power 1 mT; modulation frequency 100 kH; modulation amplitude 0.1 mT; receiver gain 2.5x102-2.5x103; scan time 240 s; time constant 1 s; and temperature 25ºC.

2.3. Chromatographic Methods

Thin Layer Chromatography (TLC) examination was carried out on 1% solution of the cephalosporins in aqueous methanol (70% v/v) using precoated silica-gel plates (Polygram Sil NHR UV 254. Mackery Nagel), with the mobile phases being an equal-part mixture (1:1) of acetone and methanol[12].

The radio-induced degradation products were determined by High Performance Liquid Chromatography (HPLC) (Agillent 1100, USA). The following validated chromatographic conditions were used during the process: Bondclone Phenyl 10μm, 30 cm x 3.90 mm column, heater: room temperature, mobile phase: 0.1 N ammonium acetate buffers in 30% of aqueous methanol solution, injection volume: 50 μL, and DAD detector: 270 nm.

2.4. Antimicrobial Activity Studies

Antimicrobial activities of control and irradiated preparations were performed by the microdilution broth method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (14). According to this procedure, inoculums of several microorganisms was prepared and antimicrobial activity was determined against these reference microorganisms. The results were expressed as minimum inhibitory concentrations (MIC in $\mu g.mL^{-1}$).

i. Inoculum Preparation

Before the test, each microorganism was inoculated in Mueller-Hinton broth for 2-6 h at 37°C. Microorganism concentration was adjusted to 0.5 McFarland standard (1-2 x 108 cfu.mL-1) and diluted to 5 x 10^5 cfu. mL^{-1} in each well.

ii. Microdilution Process

In the test, 96 wells of microtiter plates were used. Two-fold dilution of irradiated and control preparations were prepared in Mueller-Hinton broth in the wells of each row of microtiter plates (512±0.5 $\mu g.mL^{-1}$). Previously prepared microorganism suspensions were added to each well and the plates were incubated for 18-24 h at 35°C. MICs were defined as the lowest concentrations of the samples that inhibited visible growth of the microorganism.

3. Studies Carried out under Accelerated Conditions

In this part of the work, studies performed under normal environmental conditions were repeated this time for samples stored in a climatic chamber in open glass tubes at high temperature (40±2°C) and high relative humidity (75±5%) conditions as accelerated conditions over a period of three months to investigate possible degradation products and mechanism of irradiated powders.

RESULTS

1. Irradiation Process

Radiation source calibration results and the related values are shown in Table 1 and Figure 1.

Table 1. Time-absorbance-dose values of dosimeters

Time(Minute)	Absorbance	Time(Minute)	Dose (Gy)
Non-irradiated	0.045	0	14.73
Non-irradiated	0.045	1	61.3
0	0.096	2	107.45
0	0.1	3	153.73
1	0.264	5	246.73
1	0.267		
2	0.435		
2	0.428		
3	0.599		
3	0.597		
5	0.937		
5	0.928		

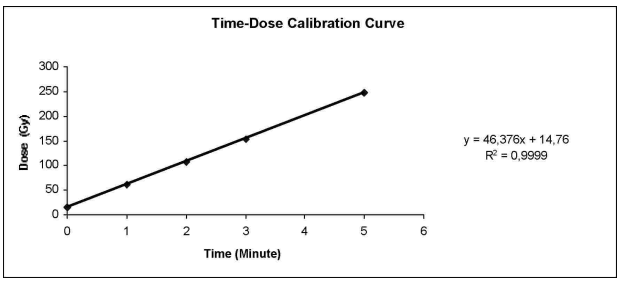


Figure 1. Time-dose calibration curve.

2. Studies Carried out under Normal Conditions

2.1. Physico-Chemical Properties

Although irradiation did not produce any change in solubility for different solvents like water, acetone, methanol and chloroform (Table 2), pH and melting point values of samples changed after irradiation at four different radiation dose levels. Results are given in Tables 3 and 4 and were compared with pharmacopeial requirements. Significant color changes were observed for all samples. The colors of the samples (when detected out of the vials) changed from white to yellow with increases in the irradiation doses.

Table 2. Measured solubility of control and irradiated samples

	Solubility ($mg.ml^{-1}$)			
	CFZ-Na			
	Water	Methanol	Chloroform	Acetone
Ref (11)	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)
N	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)
5	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)
10	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)
25	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)
40	1/1-10	1/100-1000	Insoluble(-)	Insoluble(-)

N: Non-irradiated Sample.

Table 3. Measured pH values for control and irradiated samples (n=6) ($p<0.05$)

Substance	pH					
	Ref (11)	Dose (kGy)				
		N	5	10	25	40
CFZ-Na	4-6	4.53 ±0.03	4.20 ±0.05	4.20 ±0.01	4.22 ±0.01	4.08 ±0.03

N: Non-irradiated Sample.

Table 4. Measured melting point values for control and irradiated samples (n=6) ($p<0.05$)

Substance	Melting Point (°C)					
	Ref (11)	Dose (kGy)				
		N	5	10	25	40
CFZ-Na	172	171 ±0.83	171 ±0.75	171 ±0.50	170 ±0.06	170 ±0.50

N: Non-irradiated Sample.

3.2.2. Spectroscopic Methods

For comparison, λ_{max} values determined from UV spectra of control and irradiated samples dissolved in distilled water are given in Table 5. As seen from this Table, λ_{max} values calculated for control and irradiated powder in both media were in good agreement with the values given in the Pharmacopeia[15].

Table 5. λ_{max} values calculated from UV spectra of control and irradiated samples (n=6)($p<0.05$)

Substance	λ_{max} (nm)					
	Ref (11)	Dose (kGy)				
		N	5	10	25	40
CFZ-Na	272	271.8 ±0.6	271.5 ±0.2	271.2 ±0.4	271.0 ±0.3	271.0 ±0.2

N: Non-irradiated Sample.

Control and irradiated solid samples were studied with IR, NMR and ESR spectroscopic techniques. For the IR and proton NMR analysis, no significant changes were detected for CFZ-Na samples irradiated at different dose levels (Tables 7-9).

Although control samples exhibited no ESR signal, samples irradiated at room temperature showed unresolved ESR spectra consisting of three peaks. Room temperature spectra recorded for two samples irradiated at two doses are given in Fig. 2(b) and 2(c) with the assigned peak numbers. The first resonance line appearing at the left of the spectrum was found to have a g value of 2.0104 and a peak to peak width of 5 G. As seen from the Figure 2, increase in the absorbed dose caused more intense spectra without creating change in pattern.

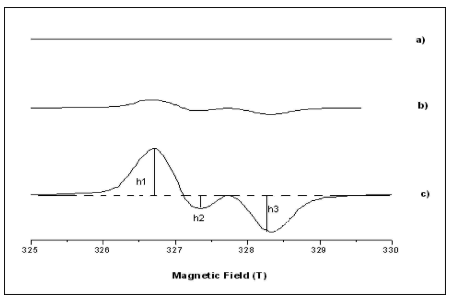


Figure 2. Room temperature ESR spectra of gamma irradiated CFZ-Na samples with assigned peak numbers. a) Non-irradiated , b) 5 kGy, c) 40 kGy. Peak heights: h1, h2, h3.

Variations in the peak heights, measured with respect to base line of these resonance lines according to applied radiation dose were studied and are presented in Figure 3. The increase in the peak to peak signal height according to applied dose was found to follow a linear curve in the 0-40 kGy dose range.

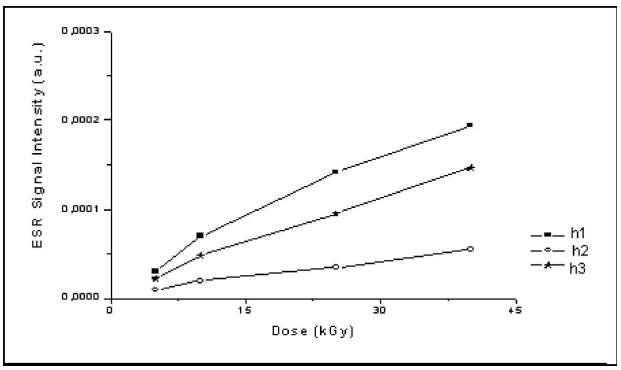


Figure 3. Variation in peak heights with absorbed radiation dose. Peak heights: h1, h2, h3.

3.2.3. Chromatographic Methods

TLC experiments performed using the technique proposed by Jacobs[16] for identification of cephalosporins produced similar R_f values for control and irradiated samples in the applied dose ranges of 5-40 kGy (Table 10). All TLC spots were found to exhibit blue-violet color when they were illuminated with UV light at 254 nm.

In the HPLC chromatograms of solid CFZ-Na, one peak disappeared after irradiation. However, intensity of some peaks increased (Figure 4). Although these changes were detected, increasing amount of active substance met the pharmacopeial requirements ($P<0.05$). Results are given in Figure 5.

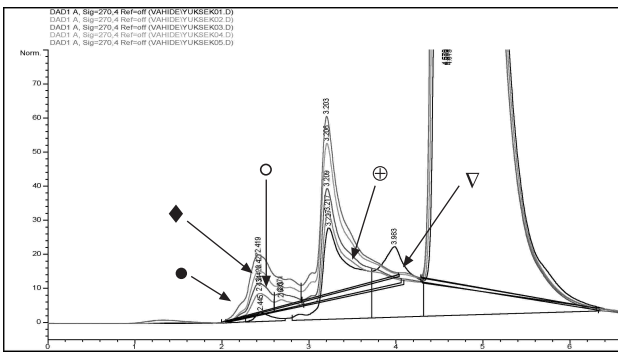


Figure 4. Peaks in non-irradiated sample (∇) and irradiated CFZ-Na at different dose rate levels (⊕ 5 k Gy, ° 10 kGy , ♦ 25 kGy , • 40 kGy).

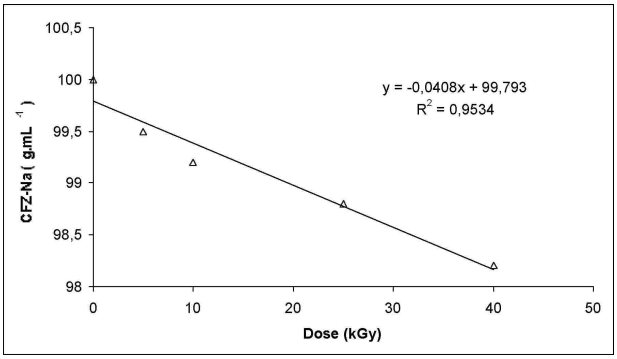


Figure 5. Radiation dose–amount of active substance curve of irradiated CFZ-Na.

3.2.4. Antimicrobial Activity Studies

In the irradiated samples, no change was observed in the activity of CFZ-Na. MIC values of control and irradiated samples are given in Table 6.

Table 6. MIC values of irradiated and control samples

Substance	MIC (µg.ml^{-1})									
	S.aureus					E.coli				
	N	5	10	25	40	N	5	10	25	40
CFZ-Na	1	1	1	1	1	4	4	4	4	4

N: Non-irradiated Sample.

3. Studies Carried out under Accelerated Conditions

For stability studies, all experiments were carried out for control and irradiated samples at four dose levels (5, 10, 25, 40 kGy) at fixed time intervals as 0, 7, 14, 21, 28, 60, and 90th days. The results obtained at the end of stability studies were similar to beginning values in irradiated and non-irradiated samples under normal conditions.

As with the results obtained in non-irradiated samples, physico-chemical properties of samples irradiated at the different dose levels did not change during the stability studies under accelerated conditions. pH values, solubility in different solvents like water, acetone, methanol and chloroform and melting points of irradiated samples changed and these changes were independent from irradiation dose levels ($p<0.05$), as with similar samples (irradiated or non-

irradiated) under normal conditions.

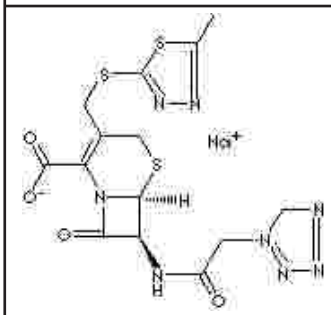
Control and irradiated solid samples were studied with IR, UV, NMR and ESR spectroscopic techniques under accelerated conditions. Although IR and NMR spectra of samples did not show any change, second λ_{max} was detected at the range of 205-210 nm after the 7th day during the UV analysis (Table 7). The data relative to the variations of in ESR signal height of different resonance lines of the 40 kGy irradiated sample over a period of 90 days is given in Figure 6. As seen from the Figure 6, the decay rate in the peak heights was very fast in the first 20 days, rather slower in the following time intervals and even at the 90th day the signal was separated from the background. The comparison of decay rates of the irradiated samples stored under normal and stability conditions showed that it was faster for the samples stored at under stability conditions[17].

Table 7. λ_{max} values calculated from UV spectra of control and irradiated samples stored at high temperature and high relative humidity in climate chamber

Time (Day)	λ_{max} (nm)				
	N	5	10	25	40
0	271	271	271	271	271
7	272 216	272 216	272 216	272 215	272 215
14	272 224	272	272	272 214	272 218
21	272 208	272 203	273 204	273 205	273
28	272 205	272	272	273	273 205
60	272 214	270	271 213	271	273 207
90	270	271	271 203	271 210	271

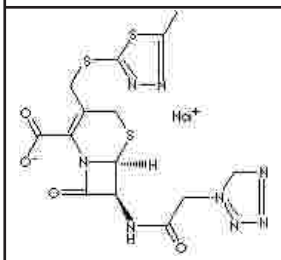
N: Non-irradiated Sample.

Table 8. IR analysis of control and irradiated samples

Active Substance and Chemical Structure	Peak Characterics	Peak (cm^{-1})					
		Dose (kGy)					
		Ref (21, 22)	N	5	10	25	40
	N-H peak	~3340	~3330	~3330	~3330	~3330	~3330
	Aliphatic C-H	~2940	~2900	~2900	~2900	~2900	~2900
	Lactam C=O	1760	1760	1762	1761	1762	1763
	Amide peak - CO-NH- - CO-NH-N - C=N-	1540-1650	1541-1667	1566	1607	1668-1663	1565
	Ester C=O	1180	1183	1184	1183	1183	1183
	C-O shift C-S shift	1050 ~2940	1064 ~2940	1062 ~2940	1062 ~2940	1062 ~2940	1061 ~2940

N: Non-irradiated Sample.

Table 9. NMR analysis of control and irradiated samples

Active Substance and Chemical Structure	Protons	Chemical Shift (δ, ppm)					
			Dose (kGy)				
		Ref (21)	N	5	10	25	40
	A	8.92 (1H; singlet; tetrazole H^5)	9.65	+	+	+	+
	B	8.00 (1H; doublet; CONH)	9.40	+	+	+	+
	C	5.45 (1H; multiplet; lactam H^3)	5.40	+	+	+	+
	D	5.10 (1H; doublet; lactam H^2)	5.01	+	+	+	+
	E	4.62 (2H; singlet; CH_2CO)	4.54	+	+	+	+
	F	3.60 (2H; singlet; CH_2S)	3.65	+	+	+	+
	G	3.16 (2H; singlet; thiazoline H^6)	3.30	+	+	+	+
	H	2.35 (3H; singlet; CH_3)	2.50	+	+	+	+

N: Non-irradiated Sample.

Table 10. Measured R_f values for control and irradiated samples (n=6) (p>0.05).

Substance	R_f Value					
	Ref (12)	Dose (kGy)				
		N	5	10	25	40
CFZ-Na	4-6	0.67 ±0.05	0.68 ±0.03	0.69 ±0.05	0.68 ±0.06	0.68 ±0.07

N: Non-irradiated Sample.

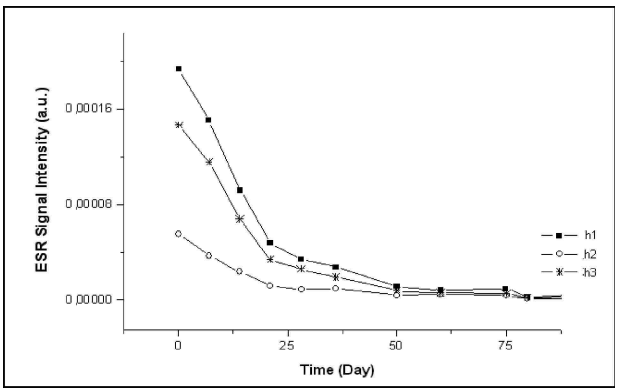


Figure 6. Variations in signal heights over a period of 90 days for CFZ-Na irradiated at a dose of 40 kGy. Peak heights: h1, h2, h3.

In the stability studies, TLC results of irradiated and control samples were unchanged. Similar results were obtained for TLC, MIC values of CFZ-Na and they did not change during the stability studies under accelerated conditions (P>0.05).

CONCLUSION

CFZ-Na is a commonly used antibiotic belonging to the semi-synthetic cephalosporin group. Its antibiotic activity is considerably decreased by heat and light, which is why sterilization of CFZ-Na requires some specific conditions. In this study, we aimed to use the gamma radiation sterilization technique as an alternative sterilization method, since it is more advantageous than conventional methods for the sterilization of CFZ-Na products.

Four different radiation dose rates were applied as 5, 10, 25 and 40 kGy. 25 kGy was chosen as the pharmacopeial-required dose. In order to observe any radiolytic change in the samples 40 kGy was employed as the accelerated/exaggerated condition. 5 and 10 kGy were the other common doses for decontamination purpose and these were the lowest conditions for the change/degradation occurring for in the antibiotics. The Sterility Assurance Level (SAL) was aimed as 10^{-6} for 25 and 40 kGy and 10^{-3}-10^{-2} for 5 and 10 kGy as mentioned in the IAEA-Report[18].

Physico-chemical properties of the irradiated (5, 10, 25 and 40 kGy) and control CFZ-Na powder samples were examined [Physicochemical properties included organoleptic properties, pH, solubility, melting point, assay of active substance and detection. Parallel to these, other tests like HPLC and TLC as chromatographic; UV, IR, NMR, and ESR as spectroscopic tests; and antimicrobial activity test as microbiological test were employed for the products stored in a climatic chamber].

During the experimental studies, the color of the samples changed from white to yellow at different radiation dose levels and color intensity increased with an increase in radiation dose from 5 kGy to 40 kGy. This observation is in agreement with the results obtained by Cruq et al.[19]. Color change in the irradiated substances is the simplest and most helpful observation to learn about possible radiolytic intermediates produced in these substances upon irradiation[20]. The color change could correspond to a chemical change of saturated compounds to unsaturated compounds. This type of radiolytic product can be formed by a disproportion of radical-radical reaction. Radical formation mechanisms as studied by ESR measurements were to be considered in the radiolysis of molecular solids as studied by Gibella et al.[5]. In this study, production of radical was supported by color change and ESR analysis. This color formation was interpreted as possibly indicating the beginning of a degradation but at the duration of the study it was within the acceptable and negligible limits of pharmacopeias. However, other experimental techniques did not show any significant changes in the characteristic properties of CFZ-Na.

Experimental results showed that solubility in different solvents did not change and were in agreement with pharmacopeial requirements or in the range of pharmacopeial requirement; pH values, melting points,

spectral properties (except ESR analysis) and R_f values were determined with some changes, but these changes were independent from irradiation dose levels ($p<0.05$). Some peak intensities increased and one of the peaks disappeared after irradiation according to HPLC analysis and the amount of active substance decreased by 98%. However, it was thought that these were the initiation of degradation at the studied radiation levels and was found that these were within the acceptable limits of pharmacopoeias[15]. MIC values of CFZ-Na also did not change after irradiation at different dose levels.

ESR dose-response curves related with the studied resonance peaks followed a linear increase in the dose range of 5-40 kGy. A model based on two tentative radical species decaying at different rates was found to explain well the experimental decay data obtained for the studied resonance peaks[17].

During the stability studies carried out under storage conditions (at 40±2°C, 75±5% relative humidity, for three months), properties of irradiated powders at 5, 10, 25 and 40 kGy did not significantly change except for λ_{max} values, and at the end of three months the active substance was observed to keep its antimicrobial activity as well. It is thought that high temperature and humidity caused these changes in λ_{max} values.

Even though irradiation caused some changes in CFZ-Na color, assay and stability studies showed that these changes were still within the acceptable limits of pharmacopoeias. Therefore, gamma radiation sterilization is a convenient technique to sterilize CFZ-Na.

Depending on the data of dose-response curve and time dependency studies, at least two different radical types might be produced upon irradiation. This result agrees with the literature[17].

According to the above results, it was concluded that CFZ-Na and/or drug delivery systems containing CFZ-Na as an active ingredient could be sterilized by gamma radiation and all these techniques can be used to monitor the effects of radiosterilization.

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