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IONIZING RADIATION INDUCED EFFECTS ON CEPHRADINE. INFLUENCE OF SAMPLE MOISTURE CONTENT, IRRADIATION DOSE AND STORAGE CONDITIONS

E.Ciranni Signoretti[1], S.Onori[2,3], L.Valvo[1], P.Fattibene[2,3], A.L. Savella[1], C.De Sena[1], S.Alimonti[1]

[1]Laboratorio di Chimica del Farmaco, [2]Laboratorio di Fisica, [3]Istituto Nazionale di Fisica Nucleare, Sezione Sanità.
Istituto Superiore di Sanità
Viale Regina Elena, 299 - 00161 Roma, Italy

ABSTRACT

The effects of gamma and electron beam irradiation on cephradine were studied as a function of dose. In particular, degradation products and free radicals induced by radiation on samples with different moisture content were investigated. The influence of daylight on the kinetics of the degradation processes was also evaluated.

INTRODUCTION

The use of ionizing radiation for the sterilization of medicinal products has steadly increased in recent years. Concomitant with the large application of this process, several studies on radiation effects in pharmaceutical systems have been performed (1-5). They are essentially focused on drugs which cannot be ster-

ilized with the conventional methods as autoclaving because of their sensitivity to high temperature. Relevant literature has been reported for cephalosporins, owing to the susceptibility of the beta-lactam ring to hydrolysis (6-8). Among these products, cephradine was found to be one of the most sensitive to radiation; effects of gamma irradiation on its bactericidal activity and physicochemical characters were investigated (7-8).

However, data from the literature do not give a wide comprehension of ionizing radiation effects on cephradine. For instance, no information about the effects induced from different radiation quality (gamma and electron beams) and from different environmental conditions during irradiation and storage are available. Moreover, among the degradation impurities due to irradiation processing, attention should be focused also on the formation of free radicals which are responsible for the biological damage. This aspect is still underdeveloped in the current literature.

Relying on the previous considerations, in this paper the influence on the degradation process of different parameters, including sample preparation, radiation quality and storage conditions was studied.

In particular, gamma irradiation of cephradine from a radioactive source and high energy electron irradiation from an accelerator were performed in well defined conditions and were controlled with suitable dosimeters. The effects of different radiation doses of both gamma and electron irradiation processes were compared. The influence of sample preparation concerning the ambiental conditions, in particular the moisture content, on the radiolytic damage was evaluated. The effect of daylight on the degradation kinetics of irradiated and unirradiated samples was also studied. Changes in cephradine structure, decrease in its purity, increase in degradation products and production of free radicals were investigated.

MATERIALS

Cephradine (7-[(amino-1,4-cyclohexadien-1-yl-acetyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid)

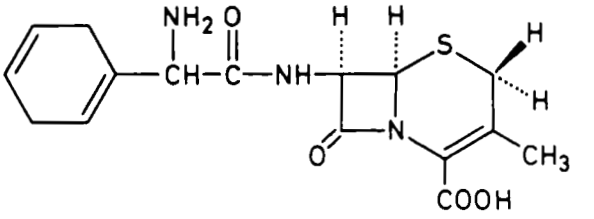

FIGURE 1
Chemical structure of cephradine.

(Fig.1) of pharmaceutical grade (from Bristol, Latina, Italy), in an anhydrous form, containing 2.9% of moisture was used.

Reagents used for liquid chromatography were: HPLC grade acetonitrile (from Merck, Darmstadt, F.T.G.), methanol (from Baker, Deventer, Holland), potassium dihydrogen phosphate (from BDH; Limited Pole England) and water previously purified by passage through a Millipore Milli-Q device. All the other materials were of analytical reagent grade.

METHODS

Sample preparation

Cephradine samples to be irradiated were put into glass (type I) tubes provided with chlorobutyl closures. The filling operations of the tubes were performed under different ambiental conditions: in nitrogen atmosphere (sample I), in the air immediately after the opening of the storage container (II) and in humidity controlled rooms at different percentage of relative humidity: 40% (III), 60% (IV), 75% (V). The moisture content of the drug, measured by thermogravimetric analysis (TGA), ranged from 2.9% (sample I) to 3.5% (sample V).

Irradiation

The samples were irradiated with electron and gamma beams at the doses of 10, 20, 30, 40 kGy (samples: $E_{10\div40}$ and $G_{10\div40}$ respectively).

A 12 MeV linear accelerator, operating at the "Istituto di Fotochimica e Radiazioni di Alta Energia" (FRAE, Bologna, Italy) was used for electron beam irradiation. The dose calibration of the accelerator was performed by 'SuperFricke' dosimetry. The dose rate at the sample position was evaluated as 3×10^6 Gy/s.

Due to the high dose rate, the sample temperature increased with dose and was monitored by a thermocouple. In addition, a forced air system was used to prevent the temperature from rising too high. In all the samples, the temperature before irradiation was within 27 ± 1°C. The final temperature, depending on dose, was in the range of $35 \div 42$°C.

As concerns gamma irradiation a cobalt-60 industrial plant (Gammaton S.p.A., Italy) was used. The dose rate was 0.4 Gy/s.

Both irradiations were performed in the $10 \div 40$ kGy range. For both the electron and gamma irradiations the dose delivered to the sample was evaluated by alanine dosimetry, developed at the Istituto Superiore di Sanità (9,10), with an uncertainty of about 3%. Three alanine pellets were used for each one of the selected dose values. They were inserted in a sample holder similar to the one used for the irradiation of cephradine. Alanine and cephradine were in turn irradiated at the same time and in the same package.

Experimental techniques

The analytical techniques adopted for detection of radiolytic damage included differential scanning calorimetry (DSC), UV spectrometry, polarimetry, high-performance liquid chromatography (HPLC), mass spectrometry and electron spin resonance (ESR) spectroscopy.

a) Melting point determinations were performed by differential scanning calorimetry, utilizing a Perkin-Elmer DGS 7 apparatus.
b) Specific optical rotation was determined at 289 nm on 0.1% w/v aqueous solutions of the irradiated and unirradiated cephradine, using a Perkin-Elmer 241 polarimeter.
c) UV spectrophotometric determinations were carried out with a Varian Cary 2200 spectrophotometer on aqueous solutions of the irradiated and unirradiated drug.

d) HPLC analyses were performed with a solvent programmer (Waters Automated Gradient Controller, equipped with two Waters Pumps, model M-6000A and M-45) and a Hewlett Packard 1040A spectrophotometer diode array detector (DAD) equipped with a HP-9000-300 computer. A Hypersil 5 μm ODS (250x4.6 mm) was utilized.

A modified method from literature (11) was used for HPLC analysis. The mobile phase for the isocratic elution was: methanol-acetonitrile-0.01M potassium dihydrogen phosphate 3:6:91 v/v/v; the measurements were performed at room temperature, at a flow rate of 1.0 ml/min, at 254 and 210 nm. A weighed quantity of each cephradine sample was dissolved in the mobile phase, in order to obtain 20 μg in the injection volume (20 μl).
Solutions of potential impurities (cephalexin, α-phenylglycine, deacetoxycephalosporanic acid) were also prepared in the same way. Mixtures of each sample and single impurities were also analysed to confirm the presence of such impurities in the main product.

For the semipreparative analysis, a 10 μm Bondapak C18 (250x 100 mm) was utilized, at a flow rate of 1.5 ml/min.

e) Mass spectrometry was performed on the trimethylsilyl (TMS)-derivatives of cephradine and its impurities.

The conversion of compounds (previously separated and collected by means of the semipreparative HPLC system) to the TMS-derivatives was carried out with BSTFA-THCS reagent (trimethylsilylfluoroacetamide-trimethylchlorosilane (4:1)).The mass spectra were recorded with a HP 5989A instrument with an ionizing electron energy of 10 eV and an ion source temperature of 250°C. The samples were introduced by direct probe after removal of silylation reagents under vacuum.

f) ESR measurements were performed at room temperature with an X band Bruker ESP 300 spectrometer equipped with a standard TE_{102} rectangular microwave cavity.

About 700 mg of cephradine powder inserted in a quartz tube was used. The amount of the sample was enough to fill completely the cavity, in order to make the results independent on the mass.

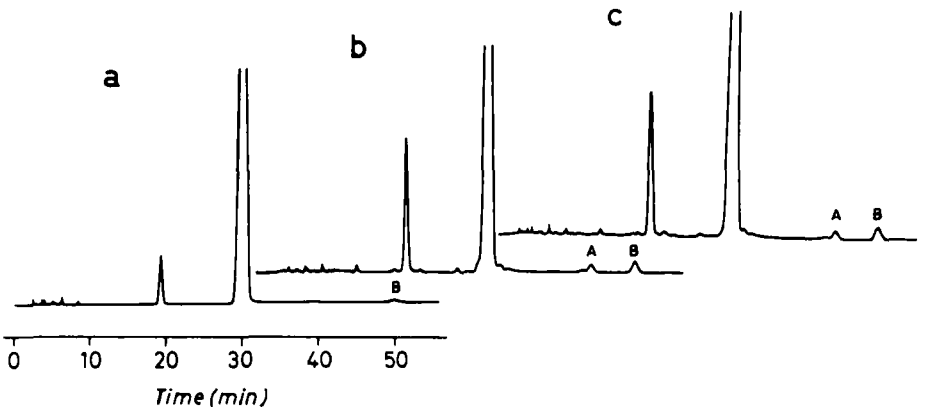

FIGURE 2

Cephradine degradation after the irradiation process. HPLC analysis at 210 nm of samples stored in darkness.
a: unirradiated sample; b: 40 kGy gamma irradiated sample (G40-V);
c: 40 kGy electron irradiated sample (E 40-V).

The following parameter setting for the ESR signal detection was used: microwave power 0.5 mW, modulation amplitude 0.2 mT, scan rate 0.6 mT/s, time constant 80 ms.

RESULTS AND DISCUSSION

Thermal behaviour and specific rotation measurements did not show any significant difference between unirradiated and irradiated cephradine, for all the examined samples.

Minimal differences among UV spectra of the various samples were found. In particular, specific absorption values, at the maximum absorbance of cephradine (260 nm), were measured as a function of radiation dose and moisture content. Within the experimental uncertainty, no dependence of the data on both the parameters can be inferred; nevertheless a systematic decrease with dose and moisture content was found. It could be partly attributed to an alteration in the O=CN-C=C linkage (12). Gamma and electron irradiated samples showed a similar behaviour.

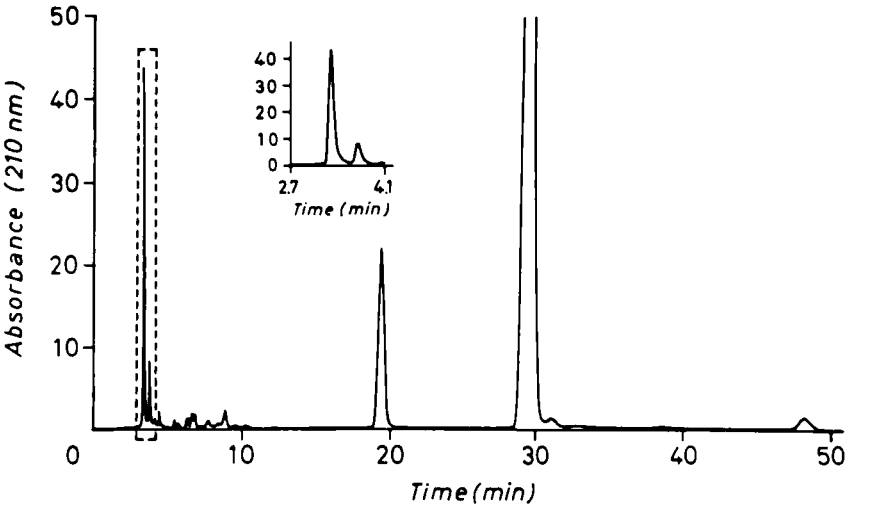

FIGURE 3

HPLC analysis at 210 nm of unirradiated cephradine, added with deacetoxycephalosporanic acid and α-phenylglycine.

HPLC analysis, performed on irradiated samples stored in darkness (Fig.2), showed degradation of cephradine after the irradiation process. An increase in the amount of impurities originally present in the unirradiated cephradine and the appearance of new degradation products were observed. The highest impurity (r.t. 19,8) corresponded to cephalexin, as reported in literature (8). Two of the products with lower retention times than the cephradine (r.t. 3,3 and 3,7) were identified as deacetoxycephalosporanic acid and -phenylglicine respectively, as results from the chromatographic profile of the unirradiated sample added with reference mixtures of the two mentioned compounds (Fig.3). The chromatograms of irradiated cephradine (Fig.2) showed also two unidentified peaks following the main compound (A, r.t. 44.3; B, r.t.50.2), which were found in a significant amount. One of them (A), absent in the unirradiated cephradine, presented a U.V. absorption profile different from that of the cephradine; the other one (B), originally present in a small quantity in the unirradiated compound, showed a spectrophotometric profile similar to that of cephradine (Fig.4).

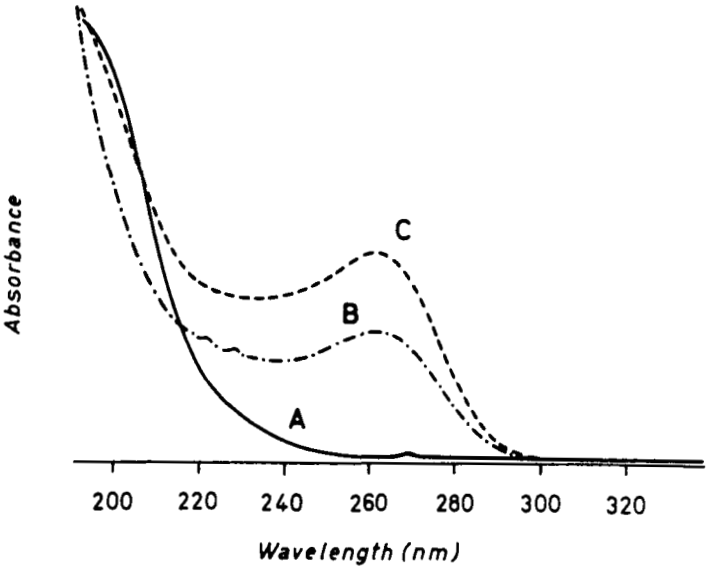

FIGURE 4

U.V. Spectrophotometric profile (HPLC diode array detector) of cephradine (C) and the two impurities A and B.

The mass spectrum of the TMS-derivative of the first compound (A) showed the most intense signals corresponding to small fragments and an intense ion at m/e 180, presumably produced by the cleavage of the α-carbonyl bond of the trimethylsilylated glycyl portion of the molecule. The mass spectrum of the TMS-derivative of the second compound (B), that showed the most intense signals corresponding to high molecular weight fragments, was more similar to the TMS cephradine spectrum than the A compound.

The influence of different parameters, such as radiation quality (gamma or electron beams), irradiation dose and moisture content of the samples on the degradation process of cephradine, determined by HPLC, is shown in figure 5. For all the tested samples, which were stored in darkness, a significant decrease in the cephradine content with dose was found. The observed differences among samples irradiated with different doses were anyway less or about 3% of the cephradine content. On the other hand, the slight

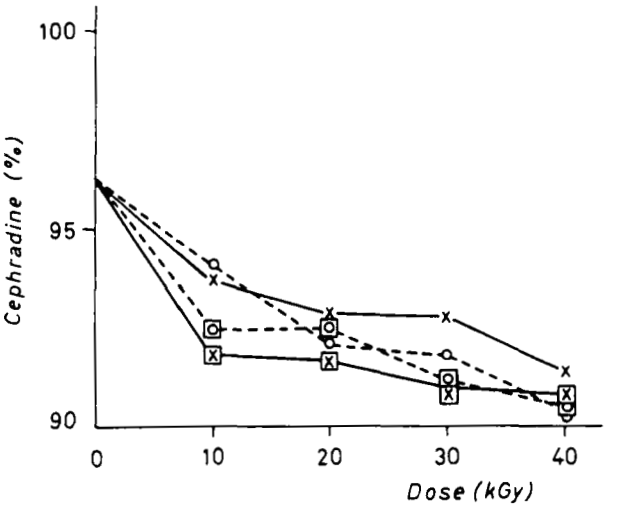

FIGURE 5

Cephradine amount decrease after irradiation, in different experimental conditions, determined by HPLC (254 nm) on samples stored in darkness.

X electron irradiated cephradine, sampled in nitrogen atmosphere (E-I),

⊠ electron irradiated cephradine, sampled in 75% ambiental humidity (E-V),

O gamma irradiated cephradine, sampled in nitrogen atmosphere (G-I),

⊙ gamma irradiated cephradine, sampled in 75% ambiental humidity (G-V).

differences found among samples exposed to different radiation quality and with different moisture content, did not put in evidence any significant influence of the two mentioned parameters on the cephradine content decrease. These HPLC quantitative data, performed at 254 nm, together with the corresponding data concerning the cephalexin increase are shown in Table 1. It should be stressed that irradiated samples, excluding some exposed at low doses, did not comply with the Italian Pharmacopoeia specifications as concerns both cephradine purity and cephalexin content (13).

After irradiation, the samples, if not stored in darkness, presented a color change from light pink to yellowish. Moreover,

TABLE 1

Cephradine Decrease and Cephalexin Increase (%±S.D.) in Gamma and Electron Irradiated Samples, at Different Doses and Humidity Content, Stored in the Dark. HPLC: 254 nm.

Sample	% Content(*) (referred to the anhydrous substance)	
	Cephradine(a)	Cephalexin(b)
R (unirradiated)	96.42±0.81	2.66±0.03
E 10-I; G 10-I	93.70±1.03 ; 94.11±0.98	4.85±0.07 ; 4.02±0.06
E 20-I; G 20-I	92.82±1.20 ; 92.10±0.84	5.01±0.05 ; 4.75±0.04
E 30-I; G 30-I	92.89±0.91 ; 91.71±1.01	5.12±0.05 ; 6.00±0.08
E 40-I; G 40-I	91.43±0.98 ; 90.40±1.20	6.14±0.08 ; 6.43±0.08
E 10-V; G 10-V	91.84±1.01 ; 92.42±1.21	5.36±0.06 ; 4.17±0.04
E 20-V; G 20-V	91.73±1.22 ; 92.40±0.84	5.36±0.07 ; 5.07±0.03
E 30-V; G 30-V	90.98±0.84 ; 91.00±1.31	6.30±0.04 ; 5.65±0.06
E 40-V; G 40-V	90.68±0.81 ; 90.58±0.95	6.55±0.05 ; 7.12±0.05

(*) mean of five replications
(a) italian official purity lower limit: 92.5% (13)
(b) italian official limit: ≤5.0% (13)

the degradation process was much more evident than that found in the product continuously stored in the dark. This effect was not observable on the unirradiated cephradine. This phenomenon, presumably attributable to benzoquinone derivates (8), is evident by the comparison of figures 2 and 6, showing the HPLC chromatograms for unirradiated, electron and gamma irradiated samples, stored in the dark and after three weeks exposure to daylight, respectively.

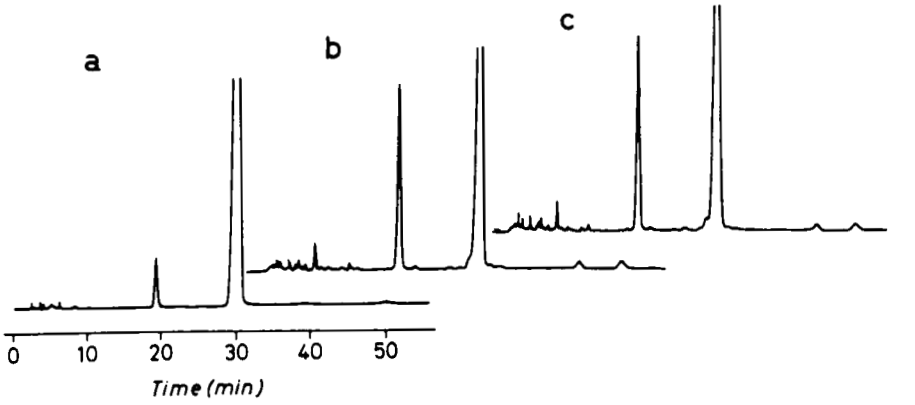

FIGURE 6

Influence of daylight exposure (3 weeks) to cephradine degradation. HPLC analysis at 210 nm.
a: unirradiated sample; b: 40 kGy gamma irradiated sample (G 40-V); c: 40 kGy electron irradiated sample (E 40-V).

No differences were found between gamma and electron irradiated samples, while unirradiated cephradine, stored in daylight, appeared colourless and showed only a negligible impurity increase. The effect of the light on the impurity level increase of irradiated samples was detected in a manifest way even after a short time (1 day) and it was found to be dose dependent. This phenomenon was very significant especially at high doses, as shown in figure 7, which is related to the kinetics degradation process of unirradiated and 20-40 kGy gamma irradiated cephradine, with the highest moisture content (G 20-V, G 40-V samples). The effect was observable also if the powder was stored in the original glass tubes which became brown after the irradiation and were only occasionally exposed to daylight.

Much attention should be paid to this effect which could lead to a misunderstanding of the irradiation induced effects on cephradine. In particular, on this basis, the very large decrease in cephradine content (40%) found by some authors (8) in irradiated samples could be explained.

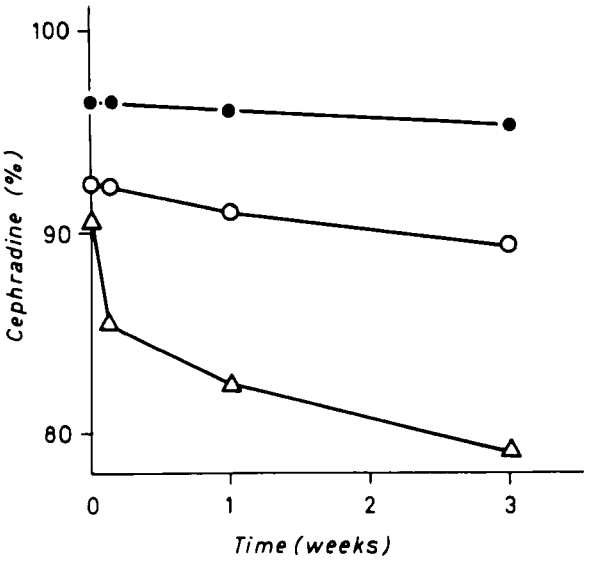

FIGURE 7

Degradation kinetic of cephradine, exposed to daylight. HPLC analysis (254 nm)
● unirradiated sample,
○ 20 kGy gamma irradiated sample (G 20-V),
△ 40 kGy gamma irradiated sample (G 40-V).

ESR measurements were carried out to investigate the presence of possible radiation induced free radicals. The unirradiated sample of cephradine did not show any signal, while stable paramagnetic centers were detectable after irradiation. A typical ESR spectrum for a gamma-irradiated sample is shown in figure 8. It appears as a center doublet and a superposed multiplet, whose number of components is difficult to guess as its outer wings are clearly seen, but the center part is covered by the large signal. The two signals have a different dependence on the microwave power; therefore they were probably originated from the interaction of the paramagnetic center with two different nuclei.

The observed spectrum is consistent with the hypothesis of nuclear hyperfine splitting due to the interaction of an unpaired

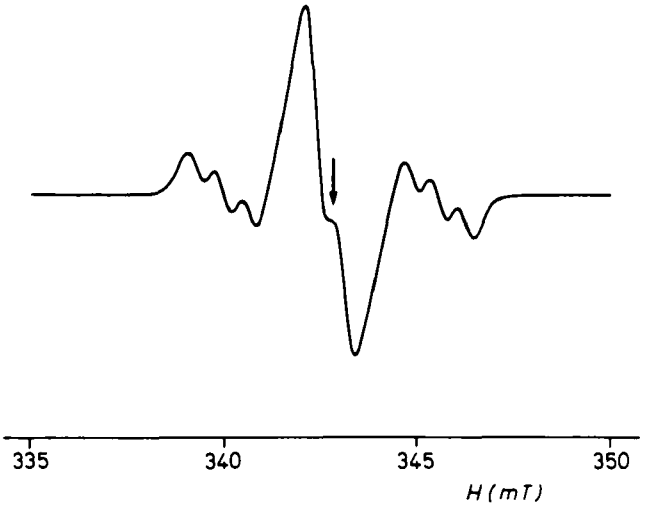

FIGURE 8

ESR spectrum of 40 kGy gamma irradiated cephradine. The arrow indicated the center of the DPPH markers resonance (g = 2.0036)

electron with a hydrogen nucleus and a NH group. Further measurements are underway to clarify the origin of the observed signal.

A qualitative analysis of the ESR spectra showed that the shape of the signal did not depend on dose, neither on radiation quality nor on relative humidity. Therefore the peak to peak amplitude of the center signal is proportional to the number of radicals produced by radiation and was used as a reliable parameter for the quantitative analysis. Its dependence on dose is shown in figure 9 for cobalt-60 irradiation. The dose-effect relationship was linear in the tested dose range. No quantitative differences between gamma and electron beam irradiated samples were observed. This result, together with the qualitative analysis, showed that for the same dose no differences were detectable in the shape or in the amplitude of the signals, that is in the nature and in the number of radicals.

Some preliminary fading measures have been performed. The detected radicals seemed to be stable enough for a quantitative

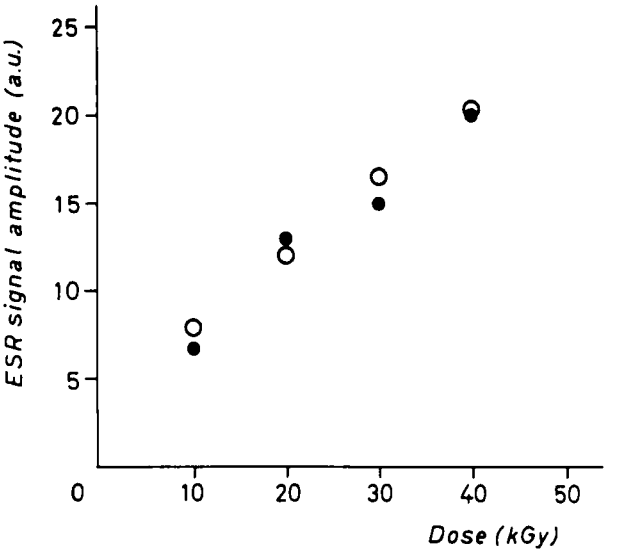

FIGURE 9

Influence of radiation dose on ESR signal peak to peak amplitude.
● electron irradiated cephradine
○ gamma irradiated cephradine

analysis on a shelf life period. In fact, in a preliminary test a well defined signal was still detectable 8 months after irradiation. Relying on the available data a half life time of about 5 months could be inferred in the conservative hypothesis of zero signal on a very long storage period. The sensitivity of the used ESR spectrometer would allow detection of a signal even after four years. A more accurate study of fading behaviour is underway; the initial results showed that no differences were detectable between samples stored in darkness or daylight for a month at room conditions.

CONCLUSIONS

Irradiation of cephradine significantly influenced the degradation process of the drug, for the formation of both the related

substances and the free radicals. The observed radiation induced effects on cephradine can be so summarized:

1) no differences on the melting point and specific optical rotation were found between irradiated and unirradiated samples; only a small decrease on the specific absorption value, measured at the maximum absorbance of cephradine, was detected;
2) on the contrary, HPLC measurements evidenced an increase in the amount of impurities already present in the unirradiated sample and the formation of new degradation products. Most of the irradiated cephradine samples did not comply with the official requirements fixed by the Italian Pharmacopoeia;
3) the observed degradative processes depended on dose and on exposure to daylight after irradiation; a slight, no significant dependence on moisture content was found. No significant differences between gamma and electron irradiated samples were also detected;
4) ionizing radiation produced free radicals whose number was proportional to the absorbed dose in the 10-40 kGy range but independent on radiation quality, moisture content and storage conditions. The radiation induced free radicals in cephradine are stable enough to be detected still several years after irradiation.

On the basis of the previous experimental evidences the irradiation of cephradine, as a possible sterilization process, is not feasible.

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