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Research article

Radiolytic studies of cefozopran hydrochloride in the solid state

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ABSTRACT

Background: The radiation sterilization is one of the best methods for sterilizing vulnerable degradation drugs like cefozopran hydrochloride.
Results: Chemical stability of radiosterylized cefozopran hydrochloride, was confirmed by spectrophotometric and chromatographic methods. EPR studies showed that radiation has created some radical defects whose concentration was no more than several dozen ppm. The antibacterial activity of cefozopran hydrochloride irradiated with a dose of 25 kGy was unaltered for Gram-positive bacteria but changed for two Gram-negative strains. The radiation sterilized cefozopran hydrochloride was not in vitro cytotoxic against human CCD39Lu normal lung fibroblast cell line.
Conclusions: Cefozopran hydrochloride in solid state is not resistant to radiation sterilization and this method cannot be used for sterilization of this compound.

1. Introduction

Cefozopran hydrochloride (CZH) is an extended spectrum fourth-generation cephalosporin with high activity against Gram-positive and Gram-negative bacteria, including methicillin-sensitive *Staphylococci, Enterococci*, and some strains of *Pseudomonas aeruginosa* [1]. CZH is often used for antibacterial prophylaxis in abdominal surgery and for treatment of postoperative intra-abdominal infections [2]. There are no results concerning infection-related mortality or severe toxicity during therapy based on CZH. Monotherapy with CZH is effective and safe for patients with febrile neutropenia [3,4]. Cefozopran is generally well tolerated in young, healthy volunteers. It does not exhibit accumulation after repeated administration. Multiple doses show similar pharmacokinetics and tissue distribution patterns to single dose administration. There is no significant effect on the pharmacokinetic properties of CZH depending on gender [5].

CZH is administered only parenterally and as all parenteral drugs must be sterile. One of the best methods for sterilization is radiation sterilization. The greatest advantage of this method is connected with the fact that it can be conducted at room or lower temperatures. It gives a great opportunity to sterilize thermolabile drugs such as CZH. CZH is instable in the solid state [6] and in solutions at increased temperature [7,8]. Therefore it should be stored in air tight containers and dissolved directly before use. To ensure safety of the therapy it should be confirmed that ionizing radiation does not change any of its pharmaceutical properties [9].

In this study, the effect of ionizing radiation on CZH in the solid phase was investigated. A standard dose of radiation sterilization (25 kGy) and higher radiation doses (50–400 kGy) were applied to provide insight into the process of CZH sterilization and also to compare the results of previous radiochemical stability studies, involving three cephems: cefoselis sulfate (CSS) [10], ceftriaxone disodium (CTD) [11] and cefpirome sulfate (CPS) [12].

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2. Experimental

2.1. Standards and reagents

CZH was obtained from CHEMOS GmbH, Regenstauf, Germany. It is a white or pale yellowish white, crystalline 98% pure powder soluble in water and conforms to the standards of Japanese Pharmacopeia XV. All other chemicals and solvents were obtained from Merck KgaA (Germany) and were of analytical grade. High-quality pure water was prepared using a Millipore Exil SA 67120 purification system (Millipore, Molsheim, France).

2.2. Methods

2.2.1. Irradiation

2.5 mg of CZH in tubes were irradiated by beta radiation in a linear electron accelerator LAE 13/9 (9.96 MeV electron beam and 6.2 μA current intensity) until they absorbed doses of 25, 50, 100, 200 and 400 kGy.

2.2.2. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded at room temperature for various times after irradiation using a Bruker ELEXSYS 500 spectrometer operating at the X-band (9.4 GHz). Detection of free radicals was performed at low microwave power (2 mW) to avoid deformation of the EPR signal by saturation effects. EPR spectra were recorded as a first derivative of microwave absorption and for free radicals with no hyperfine structure or a small value of hyperfine constant A_i (lines are overlapped) the resonance peaks appear at magnetic induction $B_r(g_i)$ if the following simple equation is fulfilled:

$$B_r(g_i) = \frac{h\gamma}{\mu_\beta g_i} \quad \text{[Equation 1]}$$

where h is the Plank constant, γ is the microwave frequency (constant), μ_β is the Bohr magneton, and g_i is the spectroscopic coefficient of radical i. The number of free radicals was obtained after double integration of EPR spectra for CZH and a comparison with the standard sample according to the procedure described elsewhere [13].

2.2.3. UV–VIS spectroscopy

Stability of radiosterilized CZH was examined using a UV/VIS Perkin Elmer Lambda 20 spectrophotometer with the UV WinLab software. 2.5 mg of each sample were dissolved in 100.0 mL of water. Spectra of obtained solutions were examined in the wavelength range of 200–400 nm.

2.2.4. HPLC analysis

To evaluate the radiostability of CZH, the Dionex Ultimate 3000 was used. Separations were performed on a Lichrospher RP-18, 5 μm, 250 mm × 4 mm. The mobile phase was a mixture composed of acetonitrile and 12 mM ammonium acetate (8:92 *V/V*). The flow rate of the mobile phase was 1.0 mL min^{-1} and the injection volume was 10 μL. The detection wavelength was 260 nm. Analyses were conducted at temp. 30°C [14].

2.2.5. Microbiological study

Minimal Inhibitory Concentration (MIC) was determined for each reference strain from the American Type Culture Collection. MIC for CPS was assayed using serial dilutions on the Mueller-Hinton liquid medium (Merck, Germany). In that experiment the microbial culture with standardized optical density was used. The applied method follows the standards of the National Committee for Clinical Laboratory Standards (NCCLS) [15].

Table 1
MIC values (mg $L.^{-1}$) of irradiated CZH samples.

	Microorganism	MIC (mg L^{-1})		
		0 kGy	25 kGy	400 kGy
1	***Proteus mirabilis* ATCC 12453**	**32**	**32**	**64**
2	***Klebsiella pneumoniae* ATCC 31488**	**64**	**64**	**128**
3	*Enterobacter hormaechei* ATCC 700323	128	128	128
4	***Enterobacter aerogenes* ATCC 13048**	**128**	**>256**	**>256**
5	***Enterococcus faecalis* ATTC 29212**	**256**	**256**	**>256**
6	***Escherichia coli* ATCC 25922**	**64**	**64**	**>256**
7	***Salmonella typhimurium* ATCC 14028**	**128**	**128**	**256**
8	***Salmonella enteritidis* ATCC 13076**	**128**	**128**	**256**
9	*Staphylococcus aureus* ATCC 25923	128	128	128
10	*Listeria monocytogenes* ATCC 7644	256	>256	>256
11	*Listeria ivanovii* ATTC 19119	>256	>256	>256
12	*Listeria innocua* ATTC 33090	>256	>256	>256
13	***Acinetobacter baumannii* ATCC 19606**	**128**	**256**	**>256**
14	*Pseudomonas aereuginosa* ATCC 27853	>256	>256	>256
15	***Rhodococcus equi* ATCC 6939**	**128**	**128**	**>256**
16	*Alcaligenes faecalis* ATCC 35655	>256	>256	>256
17	*Candida krusei* ATCC 14243	>256	>256	>256
18	*Candida albicans* ATTC 10231	>256	>256	>256
19	*Clostridium butyricum* ATTC 860	>256	>256	>256
20	*Clostridium difficile* ATCC 9689	>256	>256	>256

Bold selected species for which the observed changes in the value of MIC.

2.2.6. HPLC–MS/MS analysis

The mass spectrometry analysis was performed with the use of an Agilent hybride Q-TOF LC/MS G6520B system with a dual electro spray ion source and an Infinity 1290 UHPLC system consisting of a G4220A pump, a G1330B FC/ALS thermostat module, a G4226A autosampler, a G4212A diode array detector and a G1316C TCC module (Agilent Technologies, Santa Clara, USA). The chromatographic conditions were identical to those in the HPLC analysis. The MassHunter software B.04.00 was used to control the UHPLC–MS system and data acquisition.

The quadrupole time of the flight analyzer was tuned in the positive mode and the main parameters were optimized as follows: gas temperature 300°C, drying gas 10 L/min, nebulizer pressure 40 psig, and capillary volt. 3500 V, fragmentor volt. 200 V, skimmer volt. 65 V, octopole 1 RF volt. 250 V. The data were acquired in the auto MS/MS wise with the mass range of 50–1050 m/z and the acquisition rate of 1.2 spec./s. The CID energy was calculated from the formula 2 V (slope) ∗ (m/z) / 100 + 6 V (offset) and 2 precursors per cycle were selected with an active exclusion mode after 1 spectrum for 0.2 min. To ensure the accuracy of measurements, the reference mass correction was used and ions 121.0508 and 922.0097 m/z were used as lock masses.

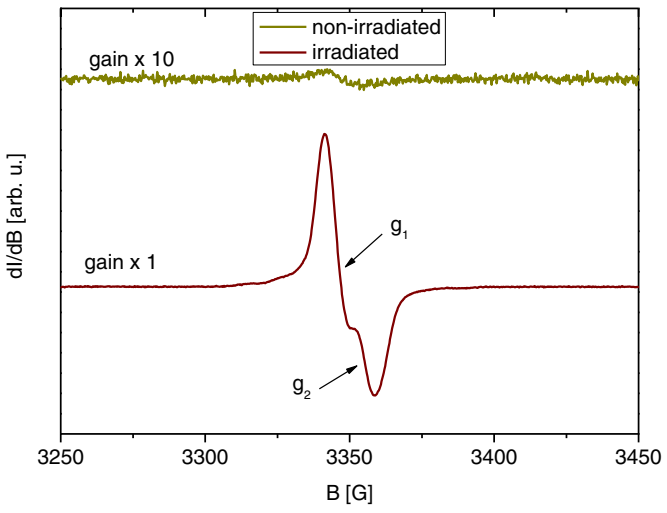

Fig. 1. EPR spectra of non-irradiated and irradiated cefozopran hydrochloride recorded 72 h after radiation sterilization (radiation dose 25 kGy).

2.2.7. Cell culture and cell viability assays

Human CCD39Lu normal lung fibroblast cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (0.1 mg mL^{-1}) and 2 mM glutamine. The cell line was cultured under standard conditions at 37°C in a humidified atmosphere comprising 95% air and 5% CO_2. To study the cytotoxic effects of radiation sterilized CZH, the CCd39Lu cells were seeded into plates at a density of 2 × 104 cells per well in 150 μL of DMEM. After the cells attached overnight, the radiation sterilized CZH at a concentration range of 0 mg L^{-1}–250 mg L^{-1} was added and MTT assay was performed after 24 h, 48 h and 72 h incubation, as described elsewhere [16].

3. Results and discussion

Forced degradation tests of fourth-generation cephalosporins showed that the β-lactam ring is an instable configuration [6,7,8,17,18,19,20,21,22]. The standard sterilizing dose (25 kGy) ensured sterility of CZH samples, but the antibacterial activity of such sterilized cefozopran hydrochloride was changed for two Gram-negative strains (*Enterobacter aerogenes* ATCC 13048 and *Acinetobacter baumannii* ATCC 19606) (Table 1). On the other, hand HPLC and UV analysis confirmed that after exposure to a standard sterilizing dose CZH was not degraded, as no significant differences were observed in chromatograms or UV spectra of non-irradiated and 25 kGy irradiated CZH samples. The microbiological method is more sensitive than HPLC or UV analysis and it should be included for quality control of antibiotics.

EPR spectra of cefozopran hydrochloride consist of two different radicals described by g-factors $g_1 = 2.0058(\pm 0.0005)$ and $g_2 = 2.0005(\pm 0.0005)$, as shown in Fig. 1. Unfortunately, because the spectra do not show any clear EPR hyperfine structure derived from hydrogen or nitrogen nuclei (hyperfine constants and therefore spin density on nuclei are small), we cannot assign individual lines to the specific types of free radicals. The concentration of free radicals for the non-irradiated sample does not exceed 0.3 ppm and this value is close to the sensitivity level of the EPR spectrometer. Immediately after radiation sterilization the concentration of radicals increased more than 100 times and reached the value of 40.8 ppm. The level of free radicals decreases exponentially vs. time after irradiation, as shown in Fig. 2 and this process is described by the following equation:

$$I(t) = I_0 + I_1 e^{-t/\tau} \quad \text{[Equation 2]}$$

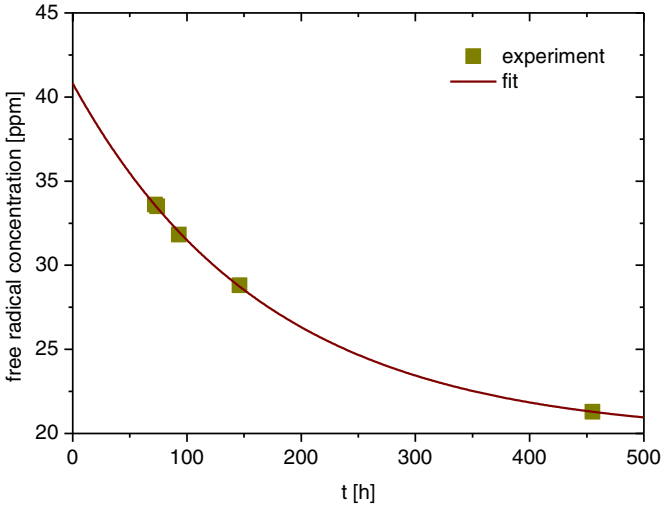

Fig. 2. Concentration of free radicals vs. time after radiation sterilization (radiation dose 25 kGy).

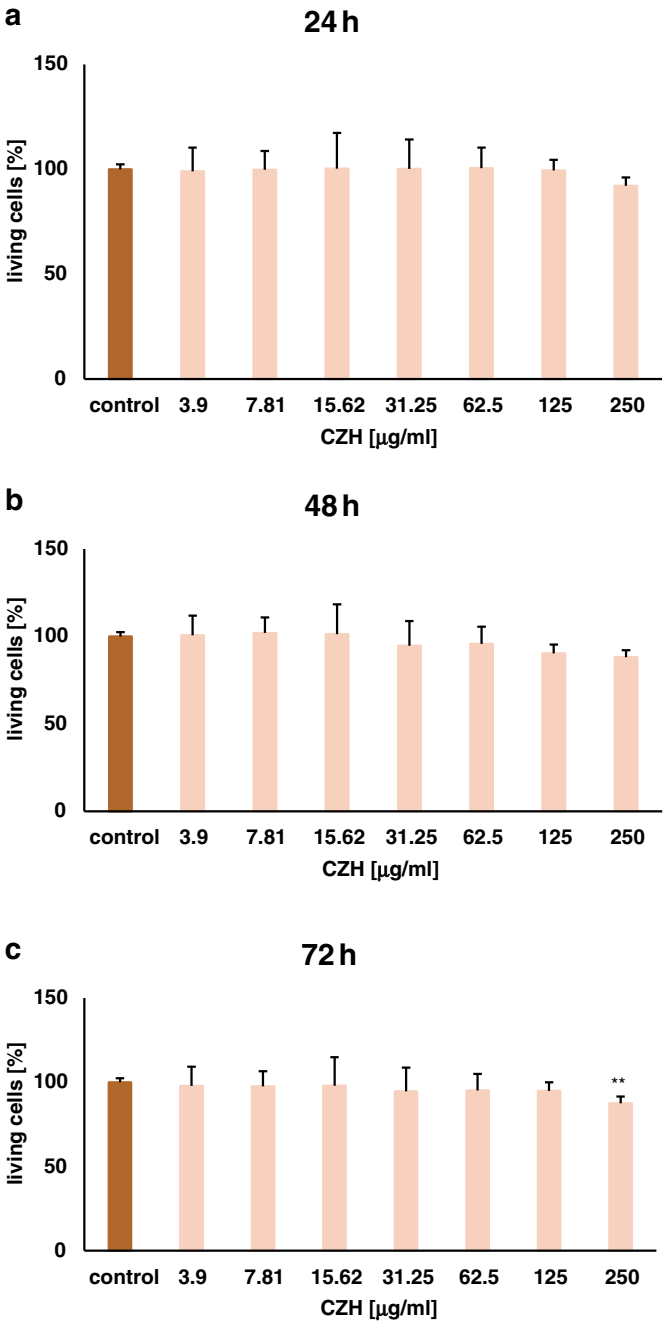

Fig. 3. Effect of radiation sterilized CZH on viability of CCD39Lu cell line after: (a) 24 h, (b) 48 h, and (c) 72 h. Results of three experiments are shown as mean ± SEM; statistically significant p value ($^{**}P < 0.01$).

Table 2
Results of quantitative analysis of CZH before and after irradiation.

Dose [kGy]	Content [%]
0	100
25	100
50	95
100	94
200	93
400	86

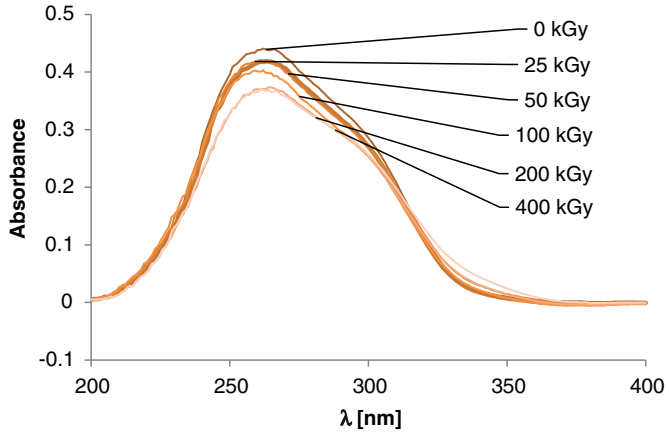

Fig. 4. UV spectra of unirradiated and irradiated CZH.

where $I(t)$ is the concentration of free radicals at any time t after radiation sterilization, $I_0 = 19.8(\pm 0.3)$ ppm is the concentration of stable radicals, $I_1 = 21(\pm 0.3)$ ppm is the concentration of unstable radicals at $t = 0$ h after irradiation, $\tau = 170(\pm 10)$ h is the mean lifetime of unstable free radicals. Equation 2 is the classical formula (slightly modified by the constant value due to the existence of stable free radicals) describing exponential decay in many processes of natural sciences: the rates of certain types of chemical reactions, radioactivity, free radical decay, heat transfer, etc.

The radiation sterilized CZH was not cytotoxic against CCD-39Lu cells after 24 h, 48 h and 72 h of incubation (Fig. 3).

Increasing radiation doses (50–400 kGy) were used to explain the process of CZH degradation and to confront previously obtained results of CSS [10], CTD [11] and CPS [12].

The CZH instability was correlated with the applied radiation dose (Table 2). UV spectra of samples irradiated with higher doses showed a slight reduction of absorbance (Fig. 4). Chromatographic analysis showed that the degradation of CZH is correlated with increasing radiation and one degradation product was observed applying that method (Table 2, Table 3, Fig. 5). HPLC-MS/MS was used to identify the degradation product of radiosterilized CZH (Table 3, Fig. 6). It was an isomer of the parent compound - CZH (Fig. 7). Isomerization of CZH was previously described [8]. The antibacterial activity of trans-CZH was lower than that of cis-CZH (parent compound) [8]. Microbiological activity of 400 kGy irradiated CZH was substantially reduced for 7 strains (Table 1).

4. Conclusions

CZH in the solid state is not resistant to radiation sterilization and this method cannot be used for sterilization of this compound. CZH is a less radiostable compound than all of the previously analyzed cephalosporins: CSS [10], CTD [11] and CPS [12].

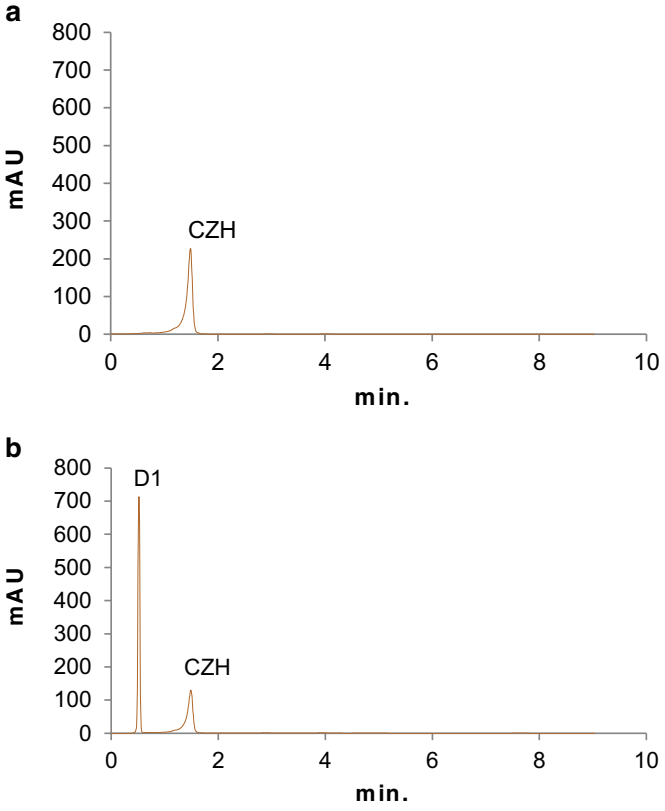

Fig. 5. HPLC-DAD chromatograms of non-irradiated (a) and irradiated (b) by dose 400 kGy CZH.

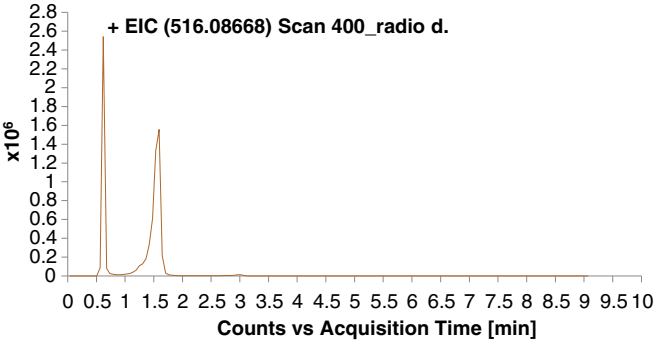

Fig. 6. HPLC-MS/MS chromatogram of irradiated by dose 400 kGy CZH.

Table 3
Exact mass measurements, elemental composition and MS/MS fragmentation of cefozopran and its radiolytic degradation product using ESI-Q-TOF method.

Label	Name	Retention time (min)	Found mass (m/z)	Theoretical mass (m/z)	Error (ppm)	Molecular formula [M + H$^+$]	MS/MS fragmentation (m/z)	Fragmentation ions formulas
1	D1	0.65	516.08290	516.08668	8.39	$C_{19}H_{18}N_9O_5S_2$	397.03290	$C_{13}H_{13}N_6O_5S_2$
							369.03788	$C_{12}H_{13}N_6O_4S_2$
							325.05244	$C_{11}H_{13}N_6O_2S_2$
							167.02137	$C_7H_7N_2OS$
							120.05035	$C_5H_8N_3$
2	CZH	1.6	516.08629	516.08668	0.83	$C_{19}H_{18}N_9O_5S_2$	397.03664	$C_{13}H_{13}N_6O_5S_2$
							369.04246	$C_{12}H_{13}N_6O_4S_2$
							325.05244	$C_{11}H_{13}N_6O_2S_2$
							167.02597	$C_7H_7N_2OS$
							120.05455	$C_5H_8N_3$

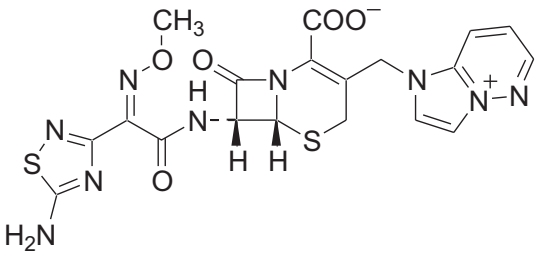

Fig. 7. Chemical structures of cefozopran.

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