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Radiostability of Florfenicol in the Solid State

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The effect of ionizing radiation on florfenicol (FF), an antibiotic with wide antibacterial properties was investigated to determine whether it can be sterilized using high-energy radiation. FF was irradiated by E-beam radiation to doses of 25 – 800 kGy, and then changes in the physico-chemical properties were examined using chromatographic methods (TLC and HPLC), spectroscopic methods (NMR and MS) and hyphenated methods (HPLC-MS). It was found that a standard sterilizing dose of 25 kGy led to the formation of two new products of radiolysis as well as lowering the content of FF by 0.95%. With higher doses of radiation, the content of FF further decreased (by 12.27% with a dose of 800 kGy), and new products of radiolysis appeared (up to five with a dose of 800 kGy). However, there were no differences between the NMR and MS spectra of irradiated and non-irradiated samples of FF. A linear dependence was found between the dose of radiation and the FF content (correlation coefficient of 0.9951) as well as between the melting point and the sum of products of radiolysis (correlation coefficient of 0.9975). It was found that a radiodegradation of FF took place by the breaking of an amide bond, leading to the formation of an aliphatic amine, which was subsequently oxidized to 4-methylsulfonylbenzoic acid. The radiolytic yield for the radiodegradation of FF was calculated to be 10.24 molecules/100 eV for a dose of 25 kGy. As a result of our investigation, we can conclude that FF shows a reasonably good radiostability in the range of doses used for sterilization, *i.e.* 25 kGy and below, and therefore it can be sterilized using high-energy radiation without changing its physicochemical, properties and hence its therapeutic efficacy.

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Introduction

Florfenicol (FF) (2,2-dichloro-*N*-{3-fluoro-1-hydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}acetamide) is a synthetic derivative of thiamphenicol.[1] It has a wide spectrum of antibacterial properties, including efficacy against Gram-positive and Gram-negative bacteria. The mechanism of action of FF, similarly to chloramphenicol and thiamphenicol, relies on slowing down the biosynthesis of bacterial proteins through reversible binding with an active center of peptydylotransferase on a subunit 50S of bacterial ribosomes. FF is not active against eucariotic ribosomes.[2,3]

The difference in the structure between florfenicol and thiamphenicol, *i.e.* the presence of a F atom at C3 instead of the hydroxyl group, protects FF against deactivation as the result of acetylation of the OH group. This reaction is catalyzed by the plasmid acquired acetylotransferase of chloramphenicol, and is one of the mechanisms most frequently responsible for antibiotic resistance of chloramphenicol derivatives.[1,4]

FF is used in the form of an injection, and therefore must be sterile. One of the methods of sterilization is ionizing radiation, which has antibacterial properties. Despite being a fast and efficient method, the exposure of drugs to ionizing radiation may lead to changes in the physicochemical properties of the drug, and therefore alter its therapeutic properties. It is therefore, necessary to determine the radiostability of a drug before using ionizing radiation as a method of sterilization.[5–7]

The changes that take place are mainly due to the production of free radicals, which can lead to chain reactions and the formation of stable products of decomposition, including optical and structural isomers. These changes take place predominantly in aqueous solutions, and the products of water radiolysis (free radicals and H_2O_2) speed up the decomposition of sterilized drugs. For this reason the method of choice is currently sterilization in the solid state. In this state drugs are far less susceptible to radiolytic damage. However, some changes can take place. The most frequently occurring effects in drugs irradiated in the solid state are: the formation of crystal-lattice defects, associated frequently with a change in the polymorphic structure, a change in the water content, color, optical rotation, the melting point temperature and the appearance of radiolysis products. These products can be formed as a result of many chemical reactions, such as oxidation, hydrolysis, deacetylation, decarboxylation, dealkylation or isomerization. These changes frequently lead to the formation of simple molecules, such as H_2, CO, CO_2, NH_3, SO_2 and H_2O.[6–12] The presence of the aforementioned changes in radiosterilized drugs does not exclude sterilization by high-energy radiation. It is important to

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quantify these changes and their effect on the therapeutical activity of the sterilized drug. The choice of appropriate analytical methods, their validation and statistical analysis are of utmost importance.[6,7]

The current work is a continuation of our research into the effects of ionizing radiation on the physicochemical properties of FF in the solid state. Previous work[13] concluded that ionizing radiation had some effect on the physicochemical properties of FF. However, these changes were observed for radiation doses significantly exceeding the sterilization dose (above 100 kGy). Higher doses of radiation led to changes in the color (from white to yellow), changes in the powder diffractogram (XRD) and a lowering of the melting point (DSC). This may suggest the formation of radiolysis products, although no changes in the FT-IR spectra or size/shape of crystals (SEM) between irradiated and non-irradiated were observed.

The aim of this work was to identify possible products of radiolysis, to investigate their structure and to explain whether their presence could lead to changes in the color of FF. That would allow us to propose a mechanism of degradation, and to compare it with the mechanism of degradation of chloramphenicol. We would also like to compare the radiostability of both compounds. In order to explore this, a number of chromatographic methods (TLC, HPLC), spectroscopic (NMR and MS) and hyphenated methods, were employed.

Methods

Materials

Florfenicol (FF) (081K680, white, loose powder, >99.0%); thiamphenicol (T0216); 4-methylsulfonylbenzoic acid (03822KE) were obtained from Sigma-Aldrich.

Method of exposure

Approximately 0.1 g of FF was placed in 5 mL of a colorless glass vial, closed with a plastic stopper and exposed to an E-beam from a linear electron accelerator, LAE 13/9 (electron beam, 9.96 MeV; current intensity, 6.2 μA) until doses of 25, 100, 400 and 800 kGy were absorbed.

Thin-layer chromatography (TLC)

We used 5.0 × 20.0 cm plates covered with silica gel Kiesegel 60 F 254. The mobile phase was *n*-hexane–ethyl acetate. A 100-μL of 1% solution FF was placed on each plate. Traces were set with a quartz lamp working at λ 254 nm. After drying, the plates were sprayed with bromocresol green or ninhydrin reagent, and dried for 10 min at 110°C.[14] Spots were characterized by the retention factor (R_f), which is defined as the distance travelled by the compound divided by the distance travelled by the solvent.

High-performance liquid chromatography (HPLC)

The HPLC system consisted of a Waters Model 616 solvent pump, equipped with a photodiode array UV-VIS Waters 996 detector set at 225 nm (corresponding to the lambda max for FF). Chromatographic separation was performed with a Waters Symmetry C18 reverse-phase column (3.9 mm × 250 mm, 2.5 μm particle size). Two different eluents were employed: phosphate buffer–acetonitrile 90:10 v/v (phase A); acetonitrile (phase B). Gradient elution with phases A and B was made at a flow rate of 1 mL per min. The following gradient program was used: 100% phase A–0% phase B for 22 min, changing to 80% phase A–20% phase B over 10 min. The separation was conducted at room temperature. The precision of the HPLC method was characterized by a relative standard deviation of 2.00%. The quantification limit was 0.61 mg L^{-1} and the limit of detection was 0.22 mg L^{-1}.

High-performance liquid chromatography–mass spectrometry (HPLC-MS)

Two HPLC-MS methods were used to analyze the products of radiolysis. One of them was described in our previous paper,[10] and in the other the Agilent 1100 instrument (Palo Alto, CA) was combined with an ion trap (IT) mass spectrometer, Model Esquire 3000 (Bruker Daltonics, Bremen, Germany). Analyses were performed on an Alltima RP-18 column (150 × 2.1 mm; Alltech), at a 0.2 ml/min flow rate; the samples were injected through a 20-μl injection loop. Elution from the column was carried out using a gradient of two solvents: A, H_2O, 0.5% formic acid, v/v; B, acetonitrile. The following elution steps were used: 0 – 5 min isocratic at 15% B, 5 – 25 min linear gradient from 15 to 40% of B, 25 – 26 min linear gradient up to 98% of B, 26 – 32 min isocratic at 100% of A. Thereafter the solvent composition returned to the starting conditions (32 – 33 min) and was kept for 40 min for the column equilibration. A mass spectrometer was equipped with an electrospray ionization (ESI) source. The IT source parameters were as follows: ESI source voltage, 4 kV; nebulization with nitrogen at 30 psi; dry gas flow, 9.0 l/min at a temperature of 310°C; skimmer 1 voltage, –15.0 V and +16.8 V in the negative and positive ion modes, respectively; collision energy set to 1 V and ramped within 40 – 400% of this value. Helium was used as a collision gas. The instrument operated under EsquireControl Ver. 5.1, and data were analyzed using a Data Analysis Ver. 3.1 package delivered by Bruker.

^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR)

Spectra were recorded at 300 K on a Bruker Avance 400 spectrometer, operating at a ^{1}H frequency of 400.13 MHz and equipped with a Bruker 5 mm QNP probe. Spectra were acquired by adding either 64 or 128 transients with an acquisition time of 3.95 s, using a 90° pulse of 7.3 s. The number of data points was 65536. There was an exponential line broadening of 0.30 Hz applied before FT. The resulting spectra were phased manually, baseline corrected, using a quadratic function and integrated manually, all using XwinNMR (Ver. 3.5 Bruker).

Mass spectrometry (MS)

The EI mass spectra were recorded on an AMD-402 two-sector mass spectrometer (AMD Intectra, Germany) with an acceleration voltage of 8 kV, an electron energy of 70 eV and an ion source temperature of 200°C.

Results and Discussion

Florfenicol was exposed to an electron beam from an accelerator to achieve the following doses: 25 kGy (standard sterilization dose), as well as 100, 400 and 800 kGy. The aim was to observe any changes taking place during the sterilisation process, and to identify any products of radiolysis. It was expected that the number of these would increase with increasing the radiation dose. When a FF sample was irradiated to 25 kGy, no radiolysis products were observed on the TLC trace. Two additional spots with $R_f = 0.80$ and 0.84 were observed for FF irradiated to 100 kGy, three for 400 kGy ($R_f = 0.05$) and four for 800 kGy ($R_f = 0.21$). The product of radiolysis with $R_f = 0.05$ was identified as 4-methylsulfonylbenzoic acid by comparing it with

Table 1 TLC results of FF before and after irradiation

Dose/kGy	R_f				
0			0.72		
25			0.72		
100			0.71	0.81	0.84[a]
400	0.05[b]		0.72	0.81	0.84[a]
800	0.05[b]	0.21	0.72	0.80	0.85[a]

a. Red spot after ninhydryne reagent.
b. Yellow spot after bromocresol green.

Table 2 HPLC results for FF before and after irradiation

t_R/min	Content, %				
	0 kGy	25 kGy	100 kGy	400 kGy	800 kGy
1.40	0.05	0.03	0.16	0.68	1.27
1.55[a]	0.14	0.18	0.62	1.13	1.86
2.20	—	—	0.23	0.70	1.16
3.70[a]	0.44	0.37	0.39	0.30	0.20
4.30	—	0.07	0.43	1.59	2.66
5.20	—	—	—	0.26	0.13
9.40 (FF)	99.00[b]	98.05	97.02	92.61	86.73
17.50	—	0.08	0.24	0.49	0.54
18.40	—	—	0.08	0.27	0.39
19.40	0.37	0.37	0.38	0.44	0.55

Non-irradiated FF used as reference for quantitative determination of radiolysis products because references for these radiolysis products are unavailable.
a. Reference standard was used for quantitative analysis.
b. Content declared by producer.

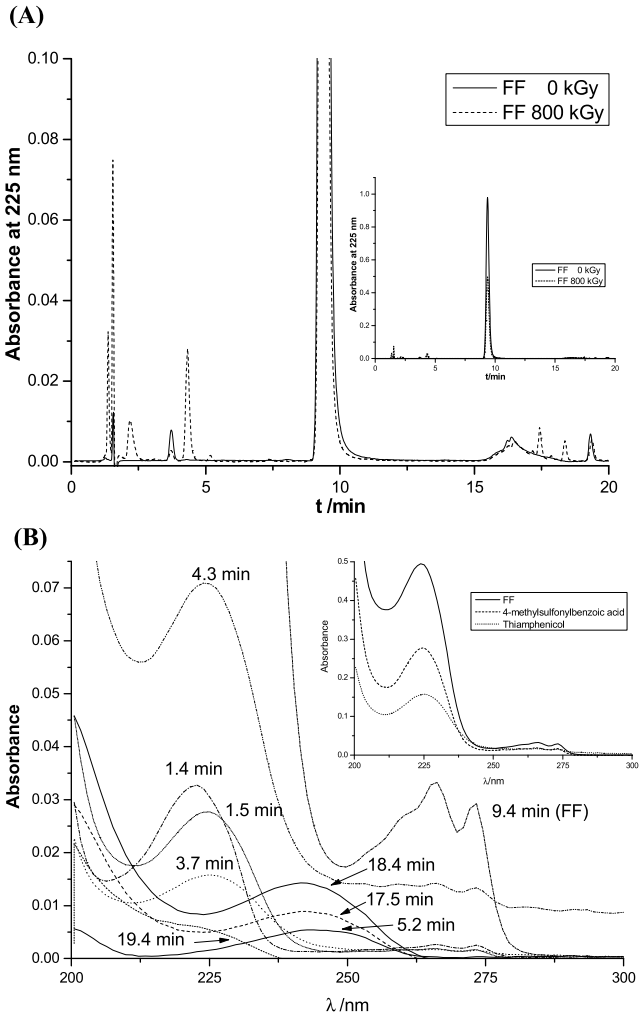


Fig. 1 (A) HPLC chromatogram for FF before and after irradiation to 800 kGy (inset figure shows total chromatogram). (B) UV spectra of radiolysis products; inset figure shows the UV spectra of FF, thiamphenicol standard, 4-methylsulfonylbenzoic acid standard.

the standard. The character of other products of radiolysis were determined by treating relevant plates with appropriate reagents (Table 1). Bromocresol green reacted with $R_f = 0.05$, thus confirming its acidic properties and the ninhydrin indicator reacted with $R_f = 0.84$, confirming its first-order amine status. The HPLC method proved to be more sensitive than TLC. The unirradiated FF showed traces of four impurities with retention times of $t_R = 1.4$, 1.55, 3.7 and 19.4 min. For a sample irradiated to 25 kGy, six peaks were observed on the HPLC trace; four of them were characterized by the same parameters as the impurities in the initial product, and the remaining two were products of the radiolysis of FF with $t_R = 4.3$ min and $t_R = 17.5$ min. For samples irradiated to 100 kGy, the number of products of radiolysis increased to four (additional two with $t_R = 2.20$ min and $t_R = 18.4$ min); for samples irradiated to 400 kGy and 800 kGy, it increased to five with the fifth showing $t_R = 5.2$ min (Fig. 1, Table 2). Quantitative HPLC analysis proved that the total amount of impurities did not exceed 1%, which confirms a statement from the producer that FF, *in substantia*, contained not less than 99% of the stated product. After irradiation to 25 and 100 kGy, the FF content was reduced by 0.95% (to 98.05%) and by 1.98% (to 97.02%), respectively. A further increase of the radiation dose caused a proportional decrease in the content of FF: for a 400-kGy dose, the content was reduced by 6.39%, and for an 800 kGy dose it was by 12.27%. With an increase of the radiation dose, an increase in the intensity of the HPLC peaks related to the radiolysis products was observed. An exception was noticed for the product with $t_R = 5.2$ min, present in samples irradiated to 400 kGy and above, the amount of which decreased with the dose, suggesting that it might be a transition or unstable product. With the help of reference samples, two impurities ($t_R = 3.7$ and 1.55 min) were identified as thiamphenicol and 4-methylsulfonylbenzoic acid. The latter was identified by TLC as a decomposition product, since it was absent in unirradiated samples, as well as in those irradiated to low doses of 25 and 100 kGy. It was observed only in samples irradiated to 400 kGy, which proves that it was a secondary, rather than the main product of radiodegradation. The HPLC method showed that its content increased with the radiation dose, confirming that it was also formed as a result of the decomposition of FF (it was also present as one of the FF impurities). The amount of thiamphenicol, present as an impurity in the original sample did not increase with the increasing dose, suggesting that FF does not degrade by breaking the C–F bond and oxidation of carbon atom in the propan-1-ol chain to hydroxyl group. Therefore, the product of radiolysis, observed on the TLC trace with $R_f = 0.84$, giving a positive reaction with ninhydrin, must be an amine containing an F atom, and could be florfenicol amine (2-amino-3-fluoro-1-[4-(methylsulfonyl)phenyl]propan-1-ol), a substance described in the literature as a main metabolite of FF.[15,16] The remaining impurities ($t_R = 1.4$ and 19.4 min) show UV spectra very similar

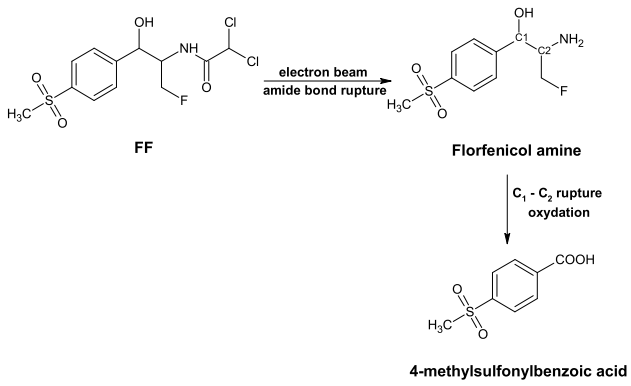


Fig. 2 Proposed scheme for the radiolytic degradation of FF.

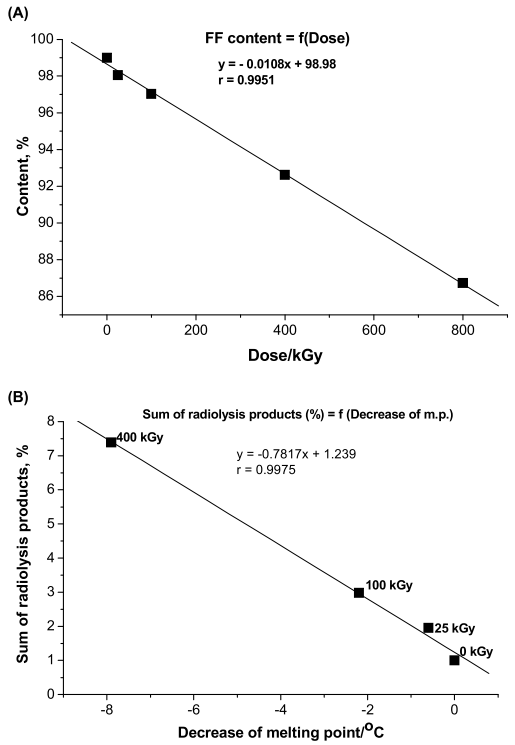


Fig. 3 Content of FF *vs.* radiation dose (A). Amount of radiolysis products *vs.* decrease of the melting point (DSC) (B).

to the original compound, and their amount increases with the dose (Table 2). They are probably products of radiolysis, being also present as impurities in the original sample. Their low content, much lower than products with t_R = 1.5 and 3.7 min, may suggest that they are products of radiolysis other than the main radiodegradation path. The product of radiolysis with t_R = 4.3 min, which gives a UV spectrum very similar to FF, represents either a main radiodegradation path, or has a very strong chromophore, because its spectrum shows the highest intensity of all decomposition products. On the other hand, the product with t_R = 2.2 min has such a low absorbance that it cannot be observed under these conditions. Possibly it is a compound of aliphatic character, and does not absorb light in the UV range investigated here. The remaining three products of radiolysis (t_R = 5.2; 17.5 and 18.4 min) show UV spectra with λ max of 242 nm, which is very different from FF. It can therefore be assumed that they are secondary products, resulting

Table 3 Radiolytic yield of FF degradation (G_{FF})

Dose/kGy	G_{FF}/molecules per 100 eV
25	10.24
100	5.33
400	4.30
800	4.13

from decomposition of the main product. Results obtained from TLC and HPLC suggest that the radiodecomposition of FF follows the path in Fig. 2. Ionizing radiation breaks the amide bond, leading to the formation of florfenicol amine (R_f = 0.84). This amine undergoes further decomposition by breaking the C1–C2 bond in the propen-1-ol chain along with the simultaneous oxidation of C1 to a carboxylic group, leading to the formation of 4-methylsulfonylbenzoic acid (R_f = 0.05 and t_R = 1.55 min) as a secondary product of radiolysis. This mechanism of the degradation is similar to radiolytic degradation of chloramphenicol. One of the products of chloramphenicol radiolysis, identified by Hong *et al.*[17] is chloramphenicol amine, which then decomposes to 4-nitrobenzoic acid. The presence of FF amine and 4-methylsulfonylbenzoic acid in irradiated samples was confirmed by HPLC-MS. Florfenicol was detected using negative ionization and, due to the presence of two Cl atoms in the molecule, its $[M–H]^-$ ions were characterized as having *m/z* 356 and 358. The first step of fragmentation was the loss of a F atom, which gave an abundant $[(M–20)–H]^-$ ion at *m/z* 336. Further fragmentation, yielding ions at *m/z* 152, 185 and 219, occurred with some internal rearrangements of the molecule. The FF amine was identified using positive ionization (ESI+), and the mass spectrum showed the presence of ions at *m/z* 248 $[M+H]^+$ and 230 $[M+H–H_2O]^+$. The 4-methylsulfonyl-benzoic acid was identified using negative ionization (ESI) and the ion of the deprotonated molecule [M–H] had *m/z* 199. The identification of all of the remaining products of radiolysis will be the subject of our next paper.[18] The results of the HPLC quantitative analysis allowed us to identify correlations between different parameters. A linear correlation (with r = 0.9951) was found between the content of FF and the dose of radiation, and also between the melting temperature and the sum of the radiolysis products (Fig. 3). The latter analysis was based on the fact that the products of radiolysis, which are also impurities, decrease the melting point of the initial compound, which confirms our earlier reports[19,20] that DSC as well as EPR can be used to monitor changes in irradiated drugs.

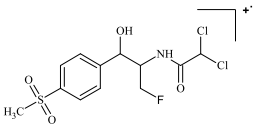
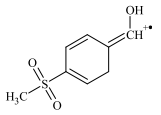
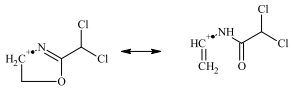
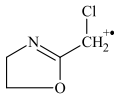
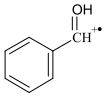
The radiolytic yield (G_{FF}) of the radiodegradation of FF, which is defined as the number of molecules of FF decomposing as a result of absorbing 100 eV of ionizing radiation,[21] was also calculated (Table 3). The smallest radiolytic effect (4.13 molecules per 100 eV) was observed for an 800 kGy dose, and was highest for 25 kGy (10.24 molecules per 100 eV). The observed decrease in the value of G_{FF} as a function of the radiation dose most likely occurs due to dissipation of the absorbed facilitating secondary reactions of radiolysis, the formation of secondary products of decomposition and the generation of free radicals and their destructive effect on the crystalline structure, as reported by us previously.[13] Similar relationships were observed for other irradiated substances.[21–26] Our results follow a trend observed by Mincher,[22] Yu[23] and Kim.[24]

In order to confirm the results obtained by chromatographic methods, MS spectra were recorded. When comparing the MS spectra of irradiated and non irradiated samples of FF, the only

Table 4 Characterization of FF mass spectra

Molecular ion	Base peak (*m/z*)	Fragment ion	Changes in the spectrum due to irradiation
358	153	186, 118, 175, 62, 77	Insignificant changes in the intensity of some ions

Table 5 Proposed structures of some fragment ions in the MS spectrum of FF

m/z	RI, %	Structure
358	0.71	
186	36.76	
153	100.00	
118	22.52	
107	20.66	

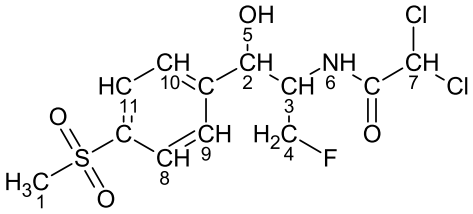
difference between the two was a small reduction in the intensity of some peaks for samples irradiated to 400 kGy. No new peak suggesting the presence of new ions was identified. The details of the MS spectra are demonstrated in Tables 4 and 5.

^{1}H-NMR spectra for irradiated and non-irradiated samples were recorded. 2D-NMR (COSY^{1}H–^{1}H) was also performed on an unirradiated sample to help with proton assignment (Table 6). No new peaks were observed, and there were no changes in the values of the chemical shifts between irradiated and non-irradiated samples.

Conclusions

On the basis of the performed experiments, we can conclude that the ionizing radiation in the range 25 – 800 kGy did not cause any changes in the MS and ^{1}H-NMR spectra of FF. Both methods appear not to be sensitive enough to study the radiostability of FF. HPLC and TLC turned out to be much more useful for allowing the products of radiolysis to be identified, separated and quantified. The two products of radiolysis that were identified were an aliphatic amine (florfenicol amine) and carboxylic acid (4-methylsulfonylbenzoic acid). Both products are structurally similar to products of the radiolysis of chloramphenicol,[17] suggesting that the radiolytic decomposition of both drugs depends neither on the type of the benzene ring substituent nor on the type of substituent on C-3 on the propan-1-ol chain, but follows the formation of the 1st order aliphatic amine in the 1st stage of the radiodegradation and carboxylic acid in the second. It is worth noting that the

Table 6 Assingment of the FF ^{1}H-NMR spectra



Chemical shift δ, ppm	Signal	Group of proton
>8 ppm	d	NH (6)
7.5 – 7.9	d-d	Aromatic (8 – 11)
6.4	s	$CHCl_2$ (7)
6.1	d	OH (5)
5.0	q	CH (2)
4.3	m	CH (3)
4.6 – 4.9	m	CH_2 (4)
3.1	m	CH_3 (1)

first stage of the radiodegradation of both drugs is analogous to their metabolism, *i.e.* both chloramphenicol amine and florfenicol amine are the main metabolites of those drugs,[1,27] eliminated from the body in the form of glucuronians and sulfates.

Both products of radiolysis that were identified are colorless, and therefore not responsible for the color change of FF samples irradiated above 25 kGy. As we suspected earlier,[13] the color change is caused by radicals trapped in the crystal lattice. This is further supported by the fact that the color disappeared when the aforementioned FF samples were dissolved and the crystal lattice is lost.

The standard radiation dose (25 kGy) caused a reduction in the content of FF by 0.95%. An increase in the radiation dose leads to a proportional decrease in the content of FF and an increase in the number of products of radiolysis. This relationship was characterized by a high correlation coefficient. This work confirms our earlier investigations on the radiostability of FF,[13] in which we stated on the basis of FT-IR, EPR, SEM, DSC and XRD experiments that a dose of 25 kGy does not cause any significant changes in the physicochemical properties of FF, and that the products of radiolysis are formed in trace amounts. However, even in this situation it would be prudent to identify all products of radiolysis using HPLC-MS and GC-MS, and to determine their toxicological profile.

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