Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 826–832

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Identification of radiolysis products of solid thiamphenicol

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ARTICLE INFO

Article history:
Received 14 November 2009
Received in revised form 20 March 2010
Accepted 22 March 2010
Available online 27 March 2010

Keywords:
Radiation sterilization
Radiolysis products
HPLC–MS
NMR
Radiodegradation

ABSTRACT

Spectroscopic and chromatographic methods (HPLC, HPLC–MS, NMR) were used to observe, separate and identify products of radiolysis of thiamphenicol (TF), irradiated in the solid state at room temperature and atmospheric pressure with an electron beam from a linear accelerator to doses between 25 and 800 kGy.

Nine products of radiolysis of thiamphenicol were identified, among them were TF amine, dichloroacetic acid, 4-methylsulfonylbenzoic acid, demono- and dedichloroderivative of TF, 2,2-dichloro-*N*-{3-hydroxy-1-[4-(methylsulfonyl)phenyl]-1-oxopropan-2-yl}acetamide and 3-({1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}amino)-3-oxopropanoic acid. The process of radiodegradation of TF was proposed as consisting of several parallel primary reactions (dehalogenation, oxidation of the OH group at C_1, hydrolysis of the amide bond, a rapture of the C_2–C_3 bond of propan-1-ol) and secondary reactions (carboxylation and oxidation).

The use of high doses, well above the sterilization dose of 25 kGy, allowed observation of changes of TF content as a function of radiation dose, calculation of radiolytic yield (G_{-TF}) and kinetic parameters of the degradation reaction. It was found that the standard sterilizing dose lowers the content of TF by only 0.1% and the radiolytic efficacy of the process of radiodegradation is 0.76 molecules/100 eV. Further increase in the dose lowers the content of TF to 92.1% for 800 kGy dose and leads to an increase in the value of G_{-TF}. It was also found that the summative process of radiodegradation of TF exposed to a beam of electrons of 10 kGy/s follows the first order reaction kinetics with a degradation constant of $k = 0.001\ s^{-1}$.

On the basis of the experiments conducted it can be stated that the radiolysis of TF in the presence of an E-beam, *in substantia*, follows multidirectional course in the same way as radiolysis of chloramphenicol. TF exposed to the standard sterilizing dose of 25 kGy degrades only by 0.1%, the amount acceptable by the ICH, and forms only one product of radiolysis (TF amine) and therefore we conclude that it can be sterilized by ionizing radiation under the conditions described above.

1. Introduction

According to the EMEA (European Agency for the Evaluation of Medicinal Products) radiation sterilization is a method of choice for thermolabile drugs. Ionizing radiation has bactericidal properties but it can also cause physicochemical changes in the sterilized drugs. These changes can affect their pharmacological activity and that was reviewed in a number of publications [1–5].

Thiamphenicol (TF) as well as chloramphenicol (CF) and florfenicol (FF), Table 1, belongs to bacteriostatic antibiotics, derivatives of 1-phenylpropan-1-ol [6] and shows activity against Gram-positive and Gram-negative microorganisms, micrococci, treponemas, rickettsia, neorickettsia and Chlamydia.

Up to now, only products of radiolysis of CF, irradiated in the solid state with gamma radiation, were identified [7,8]. In this work we have examined the process of radiolysis of TF in the solid state using E-beam as a source of ionizing radiation. Our previous work on the radiostability of TF [9] has shown that the drug has a reasonable stability up to the standard sterilizing dose of 25 kGy and the changes observed, with the exception of the formation of free radicals analyzed by EPR, were for doses higher than 25 kGy. For example, for 100 and 400 kGy doses, the differences in the XRD spectra, in the DSC curves, SEM images and the presence of radiolysis products on the TLC chromatograms were observed. To assess the observed changes quantitatively and to identify new products of radiolysis, chromatographic methods (HPLC), hyphenated (HPLC–MS) and spectroscopic methods (NMR) were used.

As in previous studies on radiochemical stability of TF [9] the drug was irradiated with the standard radiation dose (25 kGy) and with higher doses (100, 400 and 800 kGy). This was done in order

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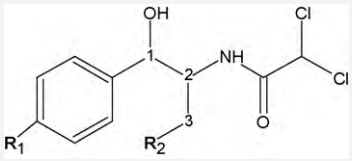
doi:10.1016/j.jpba.2010.03.026

Table 1
Antimicrobial agents derivatives of 1-phenylpropan-1-ol.



Compound	Thiamphenicol	Florfenicol	Chloramphenicol
R_1	$-SO_2CH_3$	$-SO_2CH_3$	$-NO_2$
R_2	–OH	–F	–OH
Formula	$C_{12}H_{15}Cl_2NO_5S$	$C_{12}H_{14}Cl_2FNO_4S$	$C_{11}H_{12}Cl_2N_2O_5$
Chemical name	2,2-dichloro-*N*-{1,3-dihydroxy-1-[4-(methylsulfonyl) phenyl]propan-2-yl}acetamide	2,2-dichloro-*N*-{3-fluoro-1-hydroxy-1-[4-(methylsulfonyl) phenyl]propan-2-yl}acetamide	2,2-dichloro-*N*-[1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide
Molar mass [g/mol]	356.22	358.21	323.13
Melting point [°C]	163–167	152–156	149–153

to emphasise the changes taking place, identify the products of radiolysis, estimate the radiolytic yield and kinetic parameters of degradation such as the order of the degradation reaction, the degradation constant (k) and the time taken for the 10% of TF to degrade ($t_{0.1}$).

By analysing the effect of the standard dose as well as higher doses on TF, two aims were achieved: a practical one – can E-beam be used to sterilize TF?) and a scientific one – the mechanism of the radiolytic degradation of TF.

2. Methods

2.1. Materials

TF was obtained from Sigma–Aldrich, Germany (serial number: 014K0036, >99.0%).

2.2. Method of exposure

Approximately 0.5 g of TF was placed in 5 ml colourless glass vial, closed with a plastic stopper and exposed to E-beam from a linear electron accelerator LAE 13/9 (electron beam 9.96 MeV and current intensity 6.2 μA) till they absorbed doses of 25, 100, 400 and 800 kGy. The dose rate was 10 kGy/s and temperature of the process was <35 °C.

2.3. ^1H and ^{13}C Nuclear magnetic resonance spectroscopy (^1H NMR, ^{13}C NMR)

^1H NMR spectra were recorded at 300 K on a Bruker Avance 400 spectrometer, operating at ^1H frequency of 400.13 MHz and equipped with 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 7.5 μs. Number of data points was 65536. There was an exponential line broadening of 0.30 Hz applied before FT. There resulting spectra were phased manually, baseline corrected, using a quadratic function and integrated manually all using XwinNMR (version 3.5 Bruker). DMSO-d_6 (deuterated dimethyl sulphoxide) was used as a solvent for all experiments and chemical shifts were calibrated with respect to DMSO signal at 2.51 ppm.

^{13}C NMR spectra were recorded at 300 K on Bruker Avance, operating at ^{13}C frequency of 100.62 MHz. Spectra were acquired by adding 128 transients with acquisition time of 1.36 s, using a 30° pulse of 8.2 μs. Number of data points was 65,536. Lorentzian line broadening of 1 Hz was applied before FT. The spectra were phased manually, baseline corrected, using a quadratic function. DMSO-d_6 was used as a solvent and chemical shifts were calibrated with respect to the locked DMSO-d_6 signal at 39.98 ppm.

2.4. 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 lambda max for TF). Chromatographic separation was performed with a Waters Symmetry C18 reversed-phase column (3.9 mm × 250 mm, 2.5 μm particle size). The mobile phase consisted of phosphate buffer (20 mM KH_2PO_4 in a 9:1 water–acetonitrile mixture)–water (80:20, v/v), at a flow rate of 1.0 ml min^{-1}. The separation was conducted at room temperature. The run time was 20 min. The precision of the HPLC method was characterized by relative standard deviation of 1.84%. The quantification limit was 0.42 mg L^{-1} and the limit of detection was 0.14 mg L^{-1}. The percent content was calculated as a ratio of peak area to the sum of areas of all peaks on the chromatogram. The values obtained were assigned a percent content with respect to other substances present in the mixture [10].

2.5. High performance liquid chromatography–mass spectrometry (HPLC–MS)

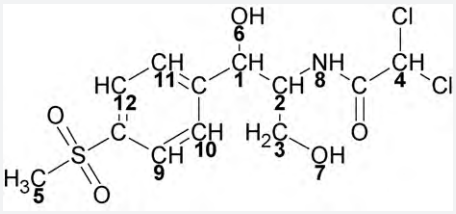
HPLC–MS measurements were performed using Prominence LC-20 (Shimadzu) liquid chromatograph coupled with tandem 4000 Q TRAP (Applied Biosystems) mass spectrometer equipped with an electrospray ion source (TurboIonSpray). Data was acquired and processed using Analyst v. 1.4.2 program. Chromatographic separation was performed with a Waters Symmetry C18 reversed-phase column (4.6 mm × 250 mm, 5 μm particle size). The mobile phase consisted of 80:20 (v/v) water–acetonitrile mixture at a flow rate of 1.0 ml min^{-1}. The separation was conducted at room temperature. The run time was 30 min. The UV chromatogram was acquired at 266 nm.

An electrospray ion source was operated at 550 °C. The capillary voltage (IS) was 4500 V in the positive ion mode and −4500 V in the negative ion mode. The declustering potential (DP) was 20 V in both modes. In the full scan experiments both positive and negative ion spectra were acquired in the same run in the 80–1000 Da mass range. Fragmentation experiments were performed using enhanced product ion (EPI) scan mode for the selected precursor ions.

3. Results and discussion

The initial sample of TF and samples irradiated to doses between 25 and 800 kGy were studied by ^1H and ^{13}C NMR. We did not observe any differences in ^{13}C NMR spectra of unirradiated sam-

Table 2
Assignment of the TF ^{1}H NMR spectra.



Chemical shift in ppm	Multiplicity	Group of protons	Changes after 800 kGy dose
8.28	d	NH (8)	• Additional signals in the range 7.67–7.95 ppm
7.60–7.95	d × d	CH aromatic (9-12)	
6.50	s	$CHCl_2$ (4)	• A new signal in the methylene range of the spectrum at 3.26 ppm
5.97	d	OH (6)	
5.05	m	CH (1)	• Disappearance of the OH peak at 4.93 ppm
4.93	t	OH (7)	
3.95	m	CH (2)	• Change in multiplicity and intensity of the OH peak at 5.97 ppm
3.60	m	CH_2 (3)	
3.16	s	CH_3 (5)	
4.05	s	Impurities	d, doublet; s, singlet t, triplet; m, multiplet d × d, two doublets

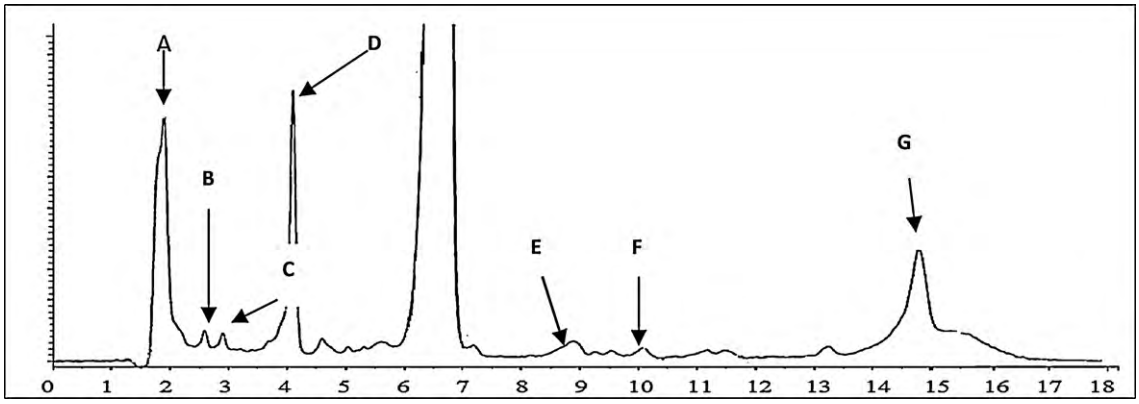
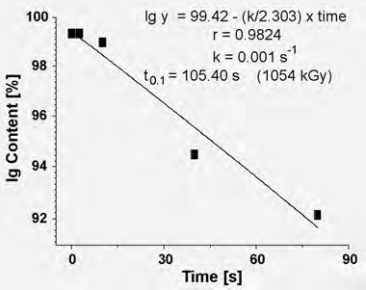


Fig. 1. HPLC–MS chromatogram (combined positive and negative ion traces) for TF before and after irradiation (A–G identified radiolysis products).

ples and the ones irradiated to 800 kGy. For the ^{1}H NMR there were no significant differences between the original sample of TF and the ones irradiated up to 400 kGy. The chemical shifts of protons in ^{1}H NMR spectrum of unirradiated TF are listed in Table 2. However, we observed some changes in the spectrum of TF irradiated to 800 kGy. Two new peaks appeared at 7.67 and 7.95 ppm, most likely due to aromatic protons of the new product/s of radiolysis. The changes in the multiplicity and intensity of the OH (6) signal at 5.97 ppm and the disappearance of the triplet due to OH (7) at 4.93 ppm could be the result of proton exchange due to the presence of dichloroacetic acid, which was identified by the HPLC–MS as one of the products of radiolysis. There was also a new peak in the methylene region of the spectrum at 3.26 ppm. One unidentified peak, probably an impurity in the original sample of TF, was present at 4.05 ppm in all samples. The changes observed in the ^{1}H NMR spectra irradiated to 800 kGy indicate that TF underwent radiolysis and therefore HPLC and HPLC–MS experiments were conducted in order to separate and identify products of radiolysis.

The stated content of the original sample of TF was 99.3% and it changed by 0.1% after irradiation with the dose of 25 kGy. As the

Table 3
Quantitative and kinetic parameters of irradiation process of TF.

Dose [kGy]	Irradiation time [s]	Content [%]	G_{-TF}		Kinetic parameters
			[molecule per 100 eV]	$[mol J^{-1}] \times 10^7$	
0	0	99.33	–	–	
25	2.5	99.23	0.76	0.79	
100	10.0	98.97	0.98	1.01	
400	40.0	94.47	3.29	3.41	
800	80.0	92.13	2.44	2.53	

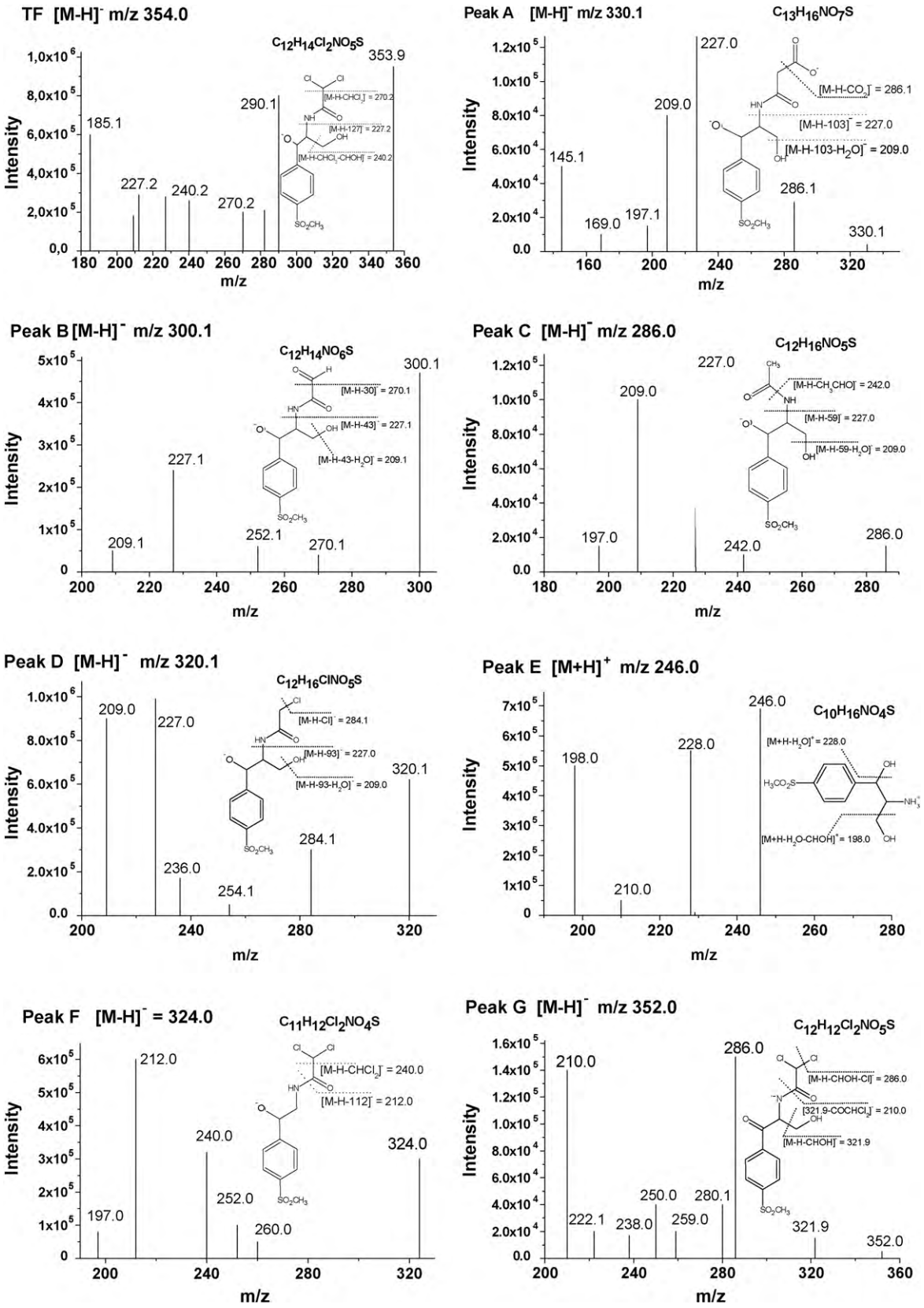


Fig. 2. CID mass spectra of $[M-H]^-$ and $[M+H]^+$ ions of TF and its radiolysis products.

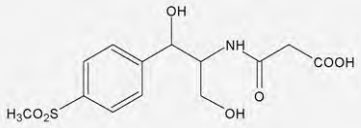
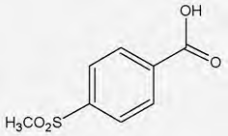
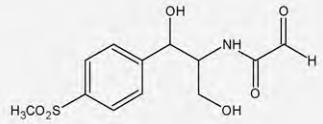
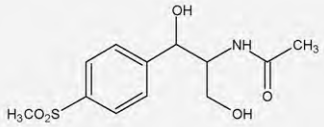
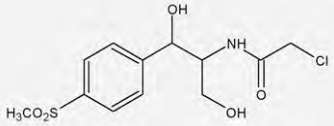
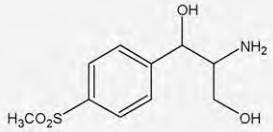
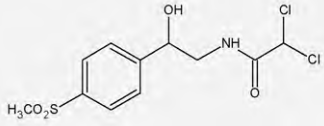
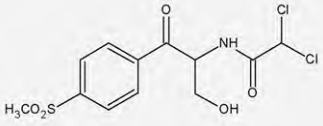
radiation dose increased, the content of TF decreased to 92.1% for 800 kGy dose. The relationship between the lg content of TF and the dose was linear with $r = 0.9824$.

As the amount of radiation absorbed by TF depends on time (10 kGy/s), we were able to study the kinetics of the degradation reaction and found that it followed first order kinetics with the rate constant $k = 1 \times 10^{-3}\ s^{-1}$ (Table 3).

On the basis of the TF content after irradiation, radiolytic yield for the process of degradation (G_{-TF}), defined as a number of molecules of TF undergoing degradation per 100 eV [11] was calculated. The lowest radiolytic yield (0.76 molecules/100 eV) was observed for 25 kGy dose (Table 3) and the highest for the dose of 400 kGy (3.29 molecules/100 eV). The initial increase in the value of G_{-TF} for doses between 25 and 100 kGy suggests the degradation of TF with fewer secondary radiolytic processes such as formation of secondary products of degradation and the destructive effect on the crystal structure. The decrease in the radiolytic yield of degradation for 800 kGy, compared with 400 kGy, could be explained by the absorbed energy being used by secondary processes, which was observed for other irradiated substances [12–16].

The HPLC–MS method was used to identify products of radiolysis (Figs. 1 and 2, Table 4). Fragment spectra of products of radiolysis were analyzed and on the basis of relative molecular masses of protonated and deprotonated molecules and fragment ions, their structures and the likely routes of mass fragmentation were proposed.

Table 4
Radiolysis products of TF identified by HPLC–MS.

Retention time [min]	Monoisotopic mass [g/mol]	Chemical structure	Chemical name
Peak A $t_R = 2.1$	127.943	$CHCl_2COOH$	Dichloroacetic acid
Peak A $t_R = 2.1$	331.073		3-({1,3-Dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}amino)-3-oxopropanoic acid
Peak A $t_R = 2.1$	200.014		4-Methylsulfonylbenzoic acid
Peak B $t_R = 2.7$	301.062		*N*-{1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}-2-oxoacetamide
Peak C $t_R = 3.0$	287.083		*N*-{1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}acetamide
Peak D $t_R = 4.3$	321.044		2-Chloro-*N*-{1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}acetamide
Peak E $t_R = 8.9$	245.072		2-Amino-1-[4-(methylsulfonyl)phenyl]propane-1,3-diol
Peak F $t_R = 10.1$	324.994		2,2-Dichloro-N-{2-hydroxy-2-[4-(methylsulfonyl)phenyl]ethyl}acetamide
Peak G $t_R = 15.0$	352.989		2,2-Dichloro-*N*-{3-hydroxy-1-[4-(methylsulfonyl)phenyl]-1-oxopropan-2-yl}acetamide

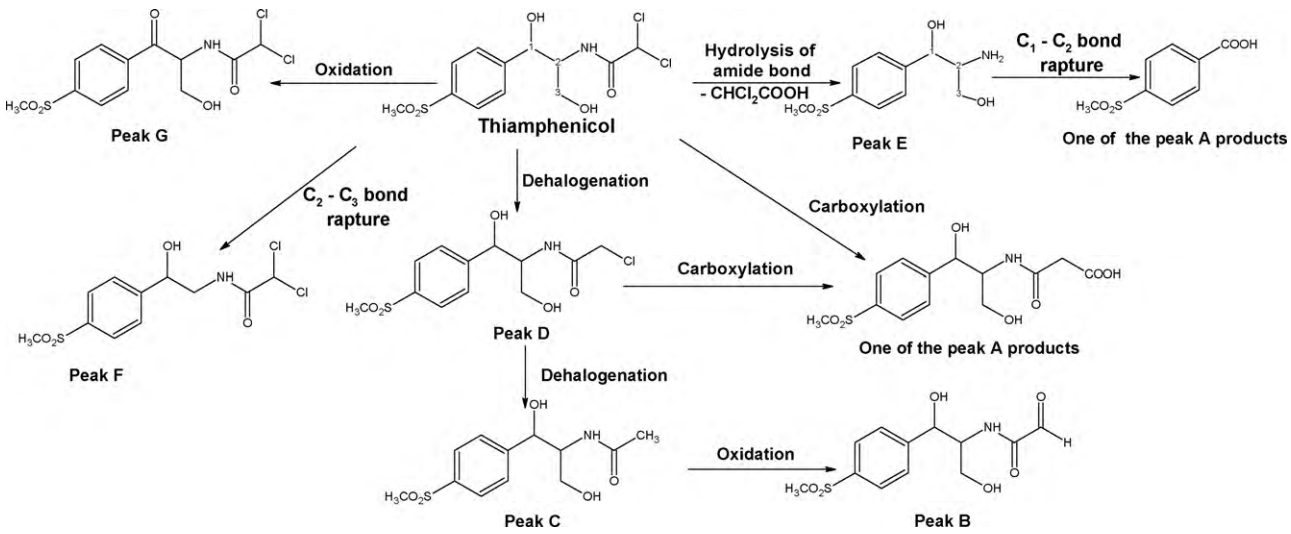


Fig. 3. Radiodegradation scheme of TF.

Collision induced dissociation (CID) mass spectrum obtained for TF (Fig. 2) indicated that the fragmentation of TF using ESI (negative ions) as a method of ionization resulted in an ion of *m*/*z* 270.2, equivalent to the loss of dichloromethane (*m*/*z* 84) and an ion with *m*/*z* 290.1, formed as a result of a loss of carbon monoxide (*m*/*z* 280) and HCl (*m*/*z* 36).

Analysis of the MS spectra (negative ions) related to the peak A (Fig. 1, t_R = 2.1 min) indicated that it resulted from three compounds with nearly identical retention times. The ratio of *m*/*z* 127.0 and its isotopic profile suggested that the main component was dichloroacetic acid. The intensity of peaks at *m*/*z* 330.1 and 199.0 was about 10 times lower. The MS spectrum of deprotonated compound with *m*/*z* 330.1 indicated that it had no chlorine atoms but had an additional carboxylic group. The formation of the COOH group as a result of ionizing radiation has been observed in the past but only in solution and for compounds with different chemical groups [17,18]. The third compound with t_R = 2.1 min turned out to be 4-methylsulfonylbenzoic acid with *m*/*z* 199.0.

Products of radiolysis C and D with t_R = 3.0 and 4.3 min had deprotonated molecular ions with *m*/*z* 286.0 and 320.1. On the basis of their fragment spectra they were identified as dedichloro- and dechloroderivatives of TF. Product B was most likely the result of oxidation of dedichloroderivate (peak C) and was a secondary product. Deprotonated ion $[M-H]^-$ of that product appeared at *m*/*z* 300.1 and MS spectrum suggested the loss of a molecule of formaldehyde to form an ion with *m*/*z* 270.1 and the loss of 2-oxoacetamide and formation of an ion with *m*/*z* 227.1.

Product E (t_R = 8.9 min) had *m*/*z* 246.0 (positive ions) and fragment ions with *m*/*z* 228.0 and 198.0. The structure of it was that of a TF amine (Fig. 2).

MS spectra of two further products of radiolysis with t_R = 10.1 and 15.0 min (products F and G) indicated the presence of chlorine atoms in their structure and their deprotonated ions were present at *m*/*z* 324.0 (product F) and *m*/*z* 352.0 (product G). Product F showed fragment ions with *m*/*z* ratio the same as TF fragment ions (*m*/*z* 240; *m*/*z* 212) and its likely structure is shown in Fig. 2. We can assume that it is formed as a result of the break of C_2–C_3 bond on the propan-1,3-diol chain. The *m*/*z* of the deprotonated ion of the product G was only two units smaller than the $[M-H]^-$ TF ion and the MS spectrum indicated that the most likely structure is the TF derivative, oxidized at C_1 position of propan-1-ol chain.

On the basis of the products of radiolysis, identified with the help of HPLC–MS method, the pathway of radiolytic degradation of TF was proposed (Fig. 3). Taking into account the intensity of each ion we can assume that the main pathways of TF degradation follow three processes: (1) the loss of one atom of chlorine and the formation of the product D, (2-chloro-*N*-{1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}acetamide), (2) the hydrolysis of amide bond, resulting in the formation of dichloroacetic acid and TF amine (one of peak A product and product E) and (3) oxidation of the hydroxylic group at C_1 on propan-1-ol chain leading to the formation of 2,2-dichloro-*N*-{3-hydroxy-1-[4-(methylsulfonyl)phenyl]-1-oxopropan-2-yl} acetamide (product G).

Analysis of TF samples exposed to doses above 100 kGy allowed us to identify minor pathways of TF degradation, which include:

- Dedihalogenation, leading to the formation of dedichloroderivative of TF (product C), which then oxidized to *N*-{1,3-dihydroxy-1-[4-(methylsulfonyl)phenyl]propan-2-yl}-2-oxoacetamide (product B),
- Carboxylation, leading to the formation of 3-({1,3-dihydroxy-1-[4-(methylsulfonyl) phenyl]propan-2-yl}amino)-3-oxopropanoic acid (one of peak A product),
- The rapture of the C_2–C_3 bond on the propan-1-ol chain (product F),
- Oxidation of TF amine to 4-(methylsulfonyl) benzoic acid (one of peak A product).

The presence of TF amine appeared after the original sample was irradiated to 25 kGy. The amount formed was below 0.1%, which is in accordance with the ICH requirements [19] for the daily dose below 2 g and therefore does not require biological activity of this degradation product to be tested. It is also necessary to add that TF amine and 4-methylsulfonylbenzoic acid are present in the body as a result of TF metabolism. They are less toxic than CF metabolites due to the presence of sulfonyl substituent on the phenyl ring. There is no need to establish toxicological profile of other products of radiolysis because their presence was only confirmed after irradiation above 100 kGy, a dose much higher than the standard sterilizing dose of 25 kGy.

To sum up, radiolysis of TF in the solid state using E-beam follows many pathways just as radiolysis of CF as a result of gamma irradiation [7,8] and the difference between the two processes is the result of percent contribution of specific reactions. Radiolysis of CF leads to the break of C_2–C_3 bond and oxidation at C_1 propan-1-ol chain. Radiolysis of TF on the other hand takes place through the hydrolysis of amide bond, as during the metabolism of this drug in

the body and demonohalogenation, a loss of one atom of chlorine. The hydrolysis of amide bond as a result of irradiation with ionising radiation was also observed for FF [20], a fluorine derivative of TF. The resulting product of radiolysis, FF amine, was oxidized to a carboxylic acid in a similar way as for TF and CF.

When comparing the processes of radiolysis of CF [7] and TF we noticed that in the case of CF most of the degradation products were already present at 25 kGy dose, whereas in the case of TF at this dose we only found TF amine to be present. The observed differences result either from the higher radiostability of TF (higher resistance to ionising radiation) or because different sources of radiation were used (gamma radiation for CF and E-beam for TF). The two types mentioned have different power at the source and therefore times taken to achieve a particular dose are different. For isotope sources, the delivered dose is of the order of kGy/h, whereas for E-beam it is of the order of kGy/s. As a consequence, the time taken for gamma sterilization is much longer (hours) than for E-beam (seconds). It is therefore possible that the processes of radiolysis can have different intensity or follow a different mechanism [21].

4. Conclusions

Our experiments confirm the results obtained previously [9] that TF can be sterilized in the solid state with a dose of 25 kGy using ionising radiation (E-beam) because the observed lowering of the content of TF is only 0.1% and TF amine, a potentially toxic product of radiolysis appears in the amounts accepted by the ICH for impurities present in medicinal products.

On the basis of HPLC–MS results we were able to identify nine, so far unpublished, products of TF radiodegradation. Three of them are secondary product (4-methylsulfonylbenzoic acid, carboxylic and didechloroderivative of TF). Five products of radiolysis identified from peaks A, D, E, F and G were structurally similar to the products of radiolysis of CF [7]. The remaining three were not mentioned in the radiodegradation studies of CF.

It was also found that the main radiodegradation pathways of TF follow the CF pathways but with different intensity. The differences observed for both drugs indicate that the degradation process depends on the *para* substitution on the phenyl ring and the type of ionising radiation used.

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