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Influence of radiation sterilization on the stability of trifluorothymidine

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Abstract

The influence of radiation sterilization on the stability of trifluorothymidine (TFT) was investigated. TFT was irradiated under ambient atmosphere with a ^{60}Co-source and with an electron accelerator at 25, 50, and 100 kGy, respectively. The radiation-induced effects were determined by chromatographic and spectroscopic methods as well as potentiometrically with a fluoride selective electrode. TFT was moderately stable to ionizing radiation. The degradation induced by electron-beam irradiation was significantly ($P = 95\%$) smaller than by γ-irradiation. The radiolysis products amounted to about 0.25% after electron-beam irradiation at 25 kGy, and to about 0.50% after γ-irradiation, respectively. The main irradiation product was 5-trifluoromethyluracil (TFMU). In addition five further impurities were detected with HPLC. Identification of degradation products was performed using HPLC-ESI-MS. A degradation path of TFT after radiation sterilization was shown.

Keywords: Trifluorothymidine determination; Degradation; γ-irradiation; Electronbeam-irradiation

1. Introduction

Radiation processing seems to be a suitable sterilization method for drug substances and final dosage forms unable to withstand heat sterilization due to it's high efficacy and to it's low costs compared to competitive methods. Moreover there are no residuals from the sterilization process. Before radiation sterilization is applicable to a drug substance or a final dosage form it has to be proven scientifically that the irradiated product is stable to ionizing radiation. The proof that the drug has unaffected potency as well as no harmful degradation products omit is required in order to apply radiation sterilization in the pharmaceutical industry. Generally, radiation-induced products have to be qualified down to a threshold of 0.1%. At lower levels, degradation products have to be identified only if they are suspected to have toxic or significant pharmacological effects (ICH Q3B, 1995).

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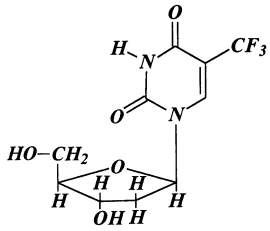

Fig. 1. Structure of TFT.

The present study shows the influence of radiation sterilization on solid trifluorothymidine (TFT). The structure is shown in Fig. 1. This drug is a highly active antitumor and antiviral agent often applied in the treatment of ocular *herpes simplex* infections. TFT is not stable in aqueous solutions and its degradation leads to several decomposition including principally 5-trifluoromethyluracil (TFMU) and the corresponding 2-deoxyribose (dR) (Nestler and Garrett, 1968; Jones, 1981). The drug is readily hydrolyzed to 5-carboxy-2′-deoxyuridine by hydroxyl ion attack (Nestler and Garrett, 1968). Aqueous TFT solutions have been irradiated adding several scavengers but the radiolysis could not be reduced to an acceptable level (Strasky, 1993). In contrast to this, no degradation products are found if TFT is stored under water-free conditions (Balansard et al., 1985). So this study will investigate if solid TFT is stable to ionizing radiation.

2. Experimental

2.1. Materials and irradiation

TFT was kindly supplied by the CIBA-Vision company (8442 Hettlingen, Switzerland).

Electron-beam irradiation was performed on a 10 MeV/150 kW-accelerator and for γ-irradiation a ^{60}Co-source was used with a dose rate of 6.9 kGy/h (Studer AG, 4658 Däniken, Switzerland).

In this work 25 kGy of absorbed radiation represented the standard sterilization dose.

2.2. Content analysis

2.2.1. Ion pair chromatography (IPC)

Instrumentation: Merck Hitachi La Chrom® (Merck KGaA, 64271 Darmstadt, Germany; Hitachi, Tokyo, Japan) consisting in a pump L-7100, an autosampler L-7200, an interface D-7000 and a diode array detector (DAD) L-7450.

Stationary phase: Stainless steel column (125 × 4 mm i.d.) packed with Supersphere RP 18 (particle size 4 μm; Merck KGaA, 64271 Darmstadt, Germany).

Mobile phase: A total of 92.5 mg hexanesulfonic acid (puriss. p.a. for ion pair chromatography, Fluka Chemicals Buchs, Switzerland) was dissolved in a mixture of 550 ml distilled water (Fistreem Cyclor™), 50ml acetonitrile (HPLC gradient grade, Merck KGaA, 64271 Darmstadt, Germany) and 5 ml glacial acetic acid (p.a grade, Scharlau Barcelona, Spain).

Flow rate: 1.0 ml/min.

Injection volume: 10 μl with the cut method (sample loop 50 μl).

Sample preparation: 10.00 mg of sample was dissolved in 100.0 ml mobile phase.

Calculation: Calculation by area at a fixed wavelength of 254 nm.

Statistical tests: Statistical tests were performed with formula F1 and F2 at a decision level of $P = 95\%$.

$$F\text{-Test: } F_{\text{cal}} = \frac{s_1^2}{s_2^2} \tag{F1}$$

$$t\text{-Test: } t_{\text{cal}} = \frac{|x1 - x2|}{s_{\text{p}}} \sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}}$$

$$\text{whereas, } s_{\text{p}} = \sqrt{\frac{(n_1 - 1) \cdot s_1^2 + (n_2 - 1) \cdot s_2^2}{n_1 + n_2 - 2}} \tag{F2}$$

2.2.2. Photometric determination

Instrumentation: UV-VIS spectrophotometer Uvikon 942, Kontron Instruments.

Cells: 10 mm macro standard quartz cells (Hellma GmbH, 79371 Müllheim, Germany).

Sample preparation: 12.50 mg of sample was dissolved with 100.0 ml distilled water (Fistreem Cyclor™). A total of 10.0 ml of this solution was diluted with water to 50.0 ml.

Acquisition wavelength: The transmission was measured at 261 nm.

Spectral bandwidth: 1 nm.

Calculations: The non-irradiated TFT was used as a standard set to 100%. All samples were correlated to the non-irradiated sample.

Statistical tests: Statistical tests were performed with Eqs. (F1) and (F2) at a decision level of $P = 95\%$

2.3. Purity analysis

2.3.1. Liquid chromatography

Instrumentation: Merck Hitachi La Chrom® (Merck KGaA, 64271 Darmstadt, Germany; Hitachi, Tokyo, Japan) consisting in a pump L-7100, an autosampler L-7200, an interface D-7000 and a diode array detector (DAD) L-7450.

Stationary phase: Stainless steel column (250 × 4 mm i.d.) packed with LiChrospher 100 RP 18 endcapped (particle size 5 μm; Merck KGaA, 64271 Darmstadt, Germany).

Mobile phase: Gradient elution consisting of (A) acetonitrile (HPLC gradient grade, Merck KGaA, 64271 Darmstadt, Germany) and (B) 20 mM phosphate buffer pH 2.5 (prepared with distilled water obtained from a Fistreem Cyclor™) as follows: 0 min 5% A–30 min 30% A.

Flow rate: 0.8 ml/min.

Injection volume: 20 μl with the cut method (sample loop 100 μl).

Sample preparation: About 5.00 mg of sample were dissolved in 1000 μl of mobile phase.

Calculation: The chromatograms were evaluated between 200 and 400 nm and calculated at a fixed wavelength of 263 nm. To calculate the amount of the impurities, the areas of the impurities were correlated to a TFT-solution corresponding to 0.5% impurities, without correction of the responses of the detected impurities.

2.3.2. Planar chromatography

Stationary phase: 20 × 10 cm HPTLC plates coated with 0.20 mm silica gel 60 F_{254} (Merck KGaA, 64271 Darmstadt, Germany), prewashed with a mixture of dichloromethane and methanol (80:20) and dried at room temperature.

Mobile phase: As mobile phase the upper phase of a mixture of ethylacetate, methanol, water and *n*-heptane (50:15:25:15) was used. The solvents were of analytical grade supplied by Scharlau (Barcelona, Spain).

Development path: 80 mm.

Chamber: Normal chamber saturated for ca. 20 min.

Sample application: Aliquots of 5 μl (60 μg) were applied on the plates (automatic application device ATS 3 (Camag, 4132 Muttenz, Switzerland) in 14 tracks of 8 mm width spaced by 5 mm using nitrogen as carrier gas.

Sample preparation: About 12.00 mg of sample was dissolved in 1000 μl methanol (HPLC gradient grade, Merck KGaA, 64271 Darmstadt, Germany).

Calculation: The measurements were performed with a Camag Scanner 3 (Camag, 4132 Muttenz, Switzerland) at a wavelength of 254 nm and data processed using Cats4 chromatography software. The calculations were made by the data-pair method. To calculate the amounts of the impurities, solutions of TFT and TFMU were applied on the same plate. The concentrations of these reference solutions corresponded to 0.2, 0.5 and 1.0% of the sample solutions, respectively.

Detection of dR: The plates were sprayed with cysteine hydrochloride reagent (10 parts acetone and 1 part of a solution (0.1 m/V%) of cysteine in 3 N sulfuric acid) heated on 95°C for 5 min and evaluated again under ultraviolet light (366 nm).

2.3.3. Determination of free fluoride

Instrumentation: The voltage was measured with a pH-Meter 654 (Metrohm AG, 9101 Herisau, Switzerland).

Measuring electrode: A fluoride ion selective crystal membrane electrode (Metrohm AG, 9101 Herisau, Switzerland) was used.

Reference electrode: Ag/AgCl reference electrode filled with 3M KCl solution purchased from Metrohm AG (9101 Herisau, Switzerland).

Sample preparation: About 50.00 mg of the samples were dissolved in 10.0 ml of a 1:1 mixture of solution TISAB (pH 5.14) and water. (TISAB = total ionic strength adjustment buffer: dissolve 58 g NaCl p.a. puriss. and 57 ml acetic acid

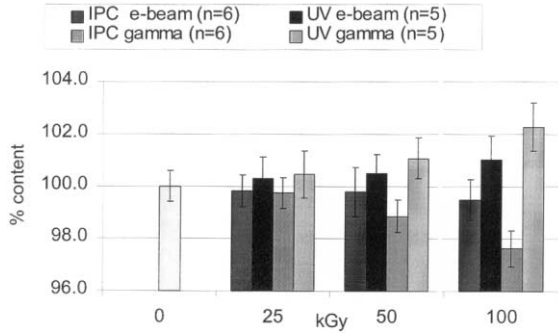

Fig. 2. Content (%) of TFT after irradiation with an electron accelerator (e-beam) and a ^{60}Co-source (gamma), determined by IPC and photometrically (UV).

glacial p.a. in 1 l water; then adjust the pH to 5–5.5 with a 5 N NaOH-solution).

2.4. HPLC-ESI-MS

HPLC-system: HPLC-ESI-MS experiments were performed on a HP1100 HPLC system (Hewlett-Packard, Palo Alto, CA). The assay was operated under the following conditions: HPLC column (Beckmann Ultrasphere ODS RP 18 250 × 2 mm); Beckmann Instruments, Fullerton, CA) maintained at 25°C; variable-wavelength detector set at 263 nm. A gradient elution was used (start conditions 5% acetonitrile and 95% water; after 30 min end conditions 30% acetonitrile and 70% water) at a constant flow rate of 0.2 ml/min. The ESI-mass spectrometer was interfaced directly to the output of the UV detector.

HPLC-ESI-MS: Negative ESI mass spectra were obtained by a Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker-Franzen GmbH, Bremen, Germany) connected to an orthogonal electrospray ion source (Hewlett-Packard, Palo Alto, CA). The MS instrument was operated under the following conditions: nitrogen nebulizer gas: 40 psi; nitrogen dry gas: 8 l/min; dry temperature: 300°C; capillary voltage: 4200 V; end plate: 3700 V; capillary exit: −96.0 V; skimmer 1: −26.1 V. The MS acquisitions were performed at the normal resolution under ion charge control (ICC) conditions (10 000) in the mass range from m/z 100 to 800 and a 32 trap drive value. To get representative mass spectra 8 scans were averaged.

3. Results and discussion

3.1. Screening tests

No visible changes were observed in the irradiated TFT. Spectroscopic methods (ultraviolet-Uvikon 942, Contron Instruments) and infrared-spectroscopy (FT-IR 2000, Perkin Elmer) as well as optical rotation (Polarimeter 241, Perkin Elmer) showed no significant differences between irradiated and non-irradiated TFT (50 kGy). Contrary, with ESR-spectroscopy radicals were found after irradiation. ESR-spectroscopy was performed with a special research instrument (Field: center field 3459.65 G, Sweep width 150.00 G, resolution 1024 points; Microwave: frequency 9.70 GHz, power 2.00e + 00 mW; Receiver: mod frequency 100.0000 kHz, mod amplitude 1.055 G).

More meaningful results than these screening tests were obtained with the following assays and purity tests.

3.2. Content analysis

The results of the ion pair chromatography (IPC) and the photometric determination (UV) are presented in Fig. 2. With the photometric method the absorption increased as a function of the irradiation dose. No significant differences were found in content with both methods between non-irradiated and irradiated (25 kGy) TFT. Moreover, electron-beam irradiation induced less degradation of the drug substance than γ-irradiation. This difference was not significant at 25 kGy of absorbed dose.

3.3. Purity analysis

3.3.1. Liquid chromatography

Typical chromatograms of non-irradiated and with 50 kGy irradiated TFT are shown in Fig. 3. The maximum wavelengths of the impurities are indicated. All impurities with higher retention

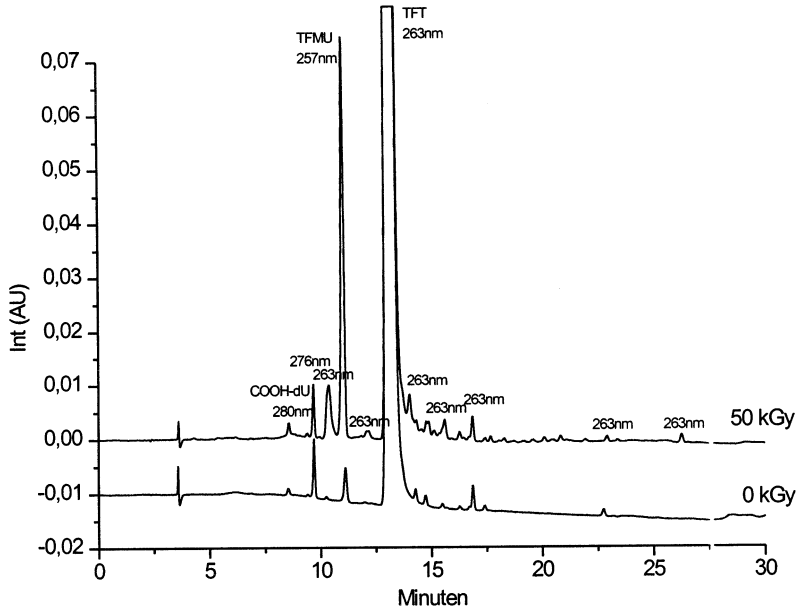

Fig. 3. Typical chromatograms of TFT non-irradiated and irradiated with 50 kGy determined by HPLC-DAD (263 nm).

times than TFT had the same absorption maximums. With shorter retention times several impurities had a changed DAD-spectrum due to a different chromophore. In addition, no further impurities were detectable in the wavelength range from 200 to 400 nm.

TFMU was the main degradation product with a maximum absorption at 257 nm. Contrary, the sugar moiety was not detectable with HPLC-DAD (263 nm). However, it was determined by TLC. TFMU and 5-carboxy-2′-deoxyuridine (COOH-dU) were identified with HPLC. The structures of other impurities were elucidated with LC-ESI-MS.

The results of the purity analysis were summarized in a sum of impurities (Fig. 4) and in impurity profiles (Figs. 5 and 6). Fig. 4 shows the sum of impurities determined by HPLC-DAD (263 nm) without response correction of the detected impurities. The sum of impurities increased in correlation to the irradiation dose. Moreover, electron-beam irradiation lead to a significant lower degradation than γ-irradiation. The sum of impurities after e-beam irradiation was about 0.27% with 25 kGy of absorbed dose. Correspondingly, after γ-irradiation it was about 0.82%.

Figs. 5 and 6 show the impurity profiles of irradiated TFT. After γ-irradiation the same impurities were generated in a higher amount than after electron-beam irradiation. The major product TFMU was about half of the sum of impurities. It was the only product that amounted to more than 0.1% (25 kGy). Because of fast hydrolysis even at pH 2.5 the prepared sample had to be injected immediately, as TFMU found in non-irradiated TFT could stem from the synthetic pathway or during storage but it could also be due to degradation in the sample solution.

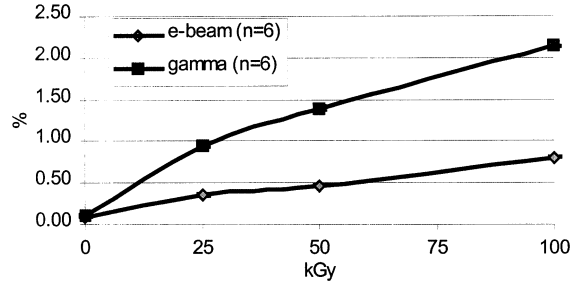

Fig. 4. Sum of impurities in TFT irradiated with an electron-accelerator (e-beam) and a ^{60}Co-source (gamma), determined by HPLC-DAD (263 nm).

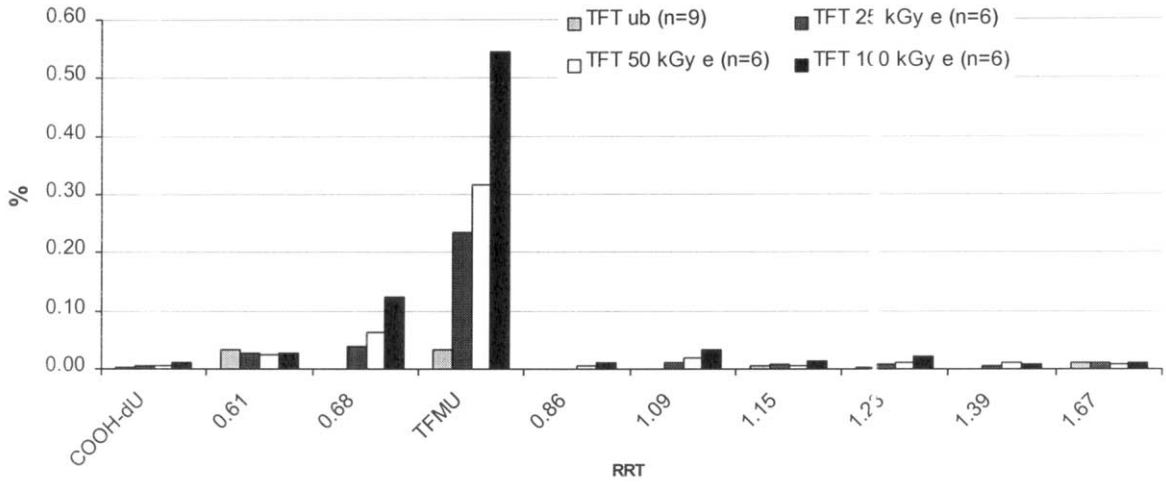

Fig. 5. Impurity profile of TFT irradiated with an electron-accelerator (e-beam) determined by HPLC-DAD (263 nm).

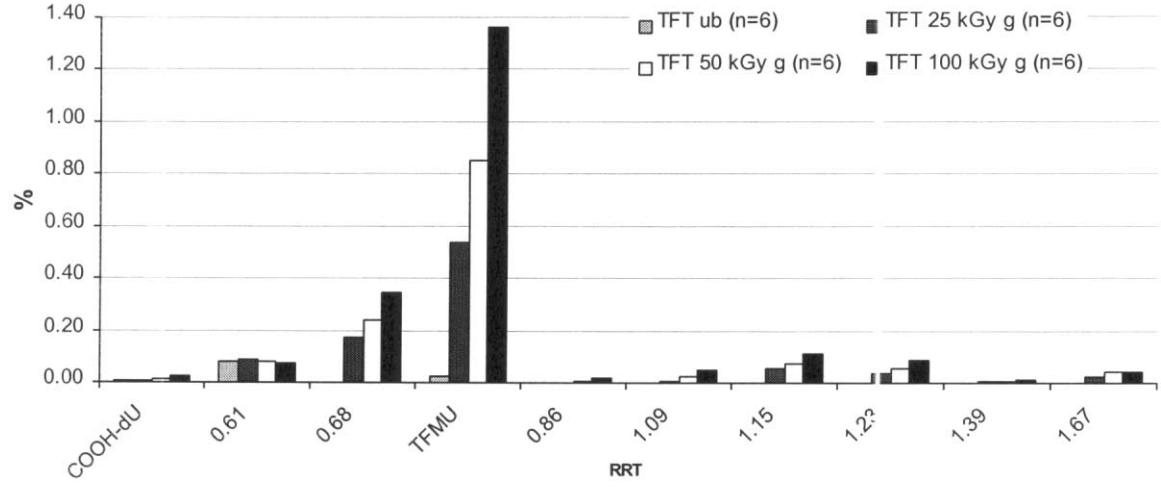

Fig. 6. Sum of impurities in TFT irradiated with a ^{60}Co-source (gamma) determined by HPLC-DAD (263 nm).

TFMU was generated in a smaller amount after dry heat treatment (5 h 140°C) than after irradiation with 25 kGy. But heat treatment at 160°C (2 h) lead to an unacceptable degradation of TFT.

Apart from TFMU, mainly one impurity (relative retention time RRT 0.68) arose significantly from irradiation. The structure of this degradation product has been elucidated by LC-ESI-MS. It generated solely by irradiation. It was neither been produced during heat treatment (5 h 140°C) nor by hydrolysis (see Section 3.4).

3.3.2. Thin layer chromatography

Fig. 7 presents typical chromatograms of non-irradiated and irradiated (50 kGy) TFT after the densitometric evaluation. The main product TFMU was well separated so it could be quantified with high precision. Compared with the HPLC-DAD method, fewer impurities were detectable.

Fig. 8 shows the sum of impurities in TFT determined by TLC. The sum of impurities increased with the irradiation dose. According to

the TLC analysis, electron-beam irradiation lead to a significant lower degradation than γ-irradiation. About 0.25% impurities arose from electron-beam irradiation with 25 kGy of absorbed radiation. After γ-irradiation with the same dose, the sum of degradation products was about 0.58%.

The impurity profiles are presented in Figs. 9 and 10. Apart from TFMU the precision of the determination was low because it was close to the quantification limit. With 25 kGy of absorbed radiation, TFMU amounted to 0.2% (e-beam) and 0.5% (gamma), respectively. The detection limit of TFT was 0.03% (20 ng detection limit/60 μg sample amount).

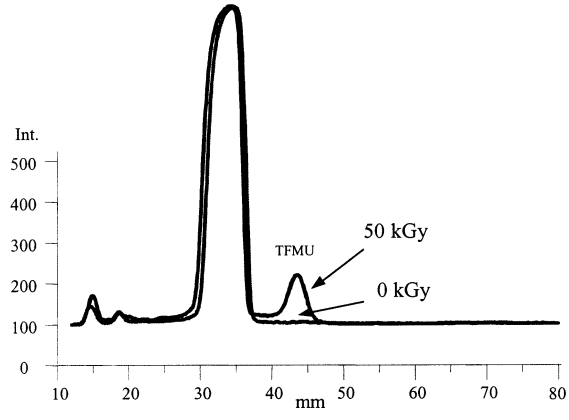

Fig. 7. TLC-chromatograms of non-irradiated and irradiated TFT (densitometric evaluation at 254 nm).

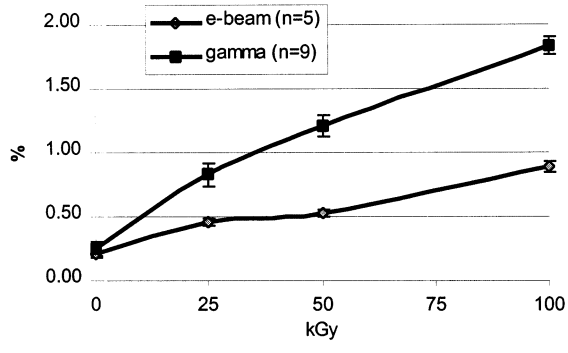

Fig. 8. Sum of impurities in TFT vs. irradiation dose after electron-beam and γ-irradiation under ambient atmosphere. The determination was performed with a TLC scanner ($\lambda =$ 254 nm).

Possible degradation products like COOH-dU and 5-carboxyuracil as well as other very polar compounds would overlay in the start spot. Therefore a more polar mobile phase (ethyl acetate, formic acid, water = 21:3:1.5) was used. With this system, neither COOH-dU (R_f 0.36) nor 5-carboxyuracil (R_f 0.49) were detected in irradiated as well as in non-irradiated TFT.

After the densitometric determination the plates were sprayed with cysteine hydrochloride reagent to detect dR and other UV-inactive compounds. Detection limits from specific reagents are listed in Table 1.

Electron-beam irradiation: dR was not detected within a limit of detection of 0.03% (20 ng detection limit/60 μg sample amount).

γ-irradiation: dR amounted to less than 0.2% with 50 and 100 kGy of absorbed radiation, respectively, and when irradiated with 25 kGy it was not detectable.

Irradiation of dR with 25, 50 and 100 kGy showed that this radiolysis product itself was very sensitive to γ-rays. The substance liquified when irradiated with doses more than 50 kGy. The degradation was proved by TLC as well as by measuring the optical rotation. In either case a significant decomposition of dR was found. So dR is assumed to decompose in irradiated TFT as well.

3.3.3. Determination of fluoride

The voltage of fluoride standard solutions was measured to determine the linear range. The calibration curve is presented in Fig. 11. The linear range was between 10^{-1} and about 10^{-6} mol/l. Concentrations below 10^{-6} mol/l were extrapolated from the data points above 220 mV.

The results of the fluoride determination is shown in Fig. 12. In the non-irradiated reference about 0.015% (mol/mol) free fluoride was found. The concentration of fluoride increased after irradiation. At 25 kGy about 0.04–0.05% free fluoride was found. The difference between electron-beam and γ-irradiation was insignificant.

These results showed that TFT was degraded by fission of C–F bonds. One possible way of degradation induced by γ-rays is producing 5-hydroxydifluoromethyl-2′-deoxyuridine that will probably break down yielding COOH-dU.

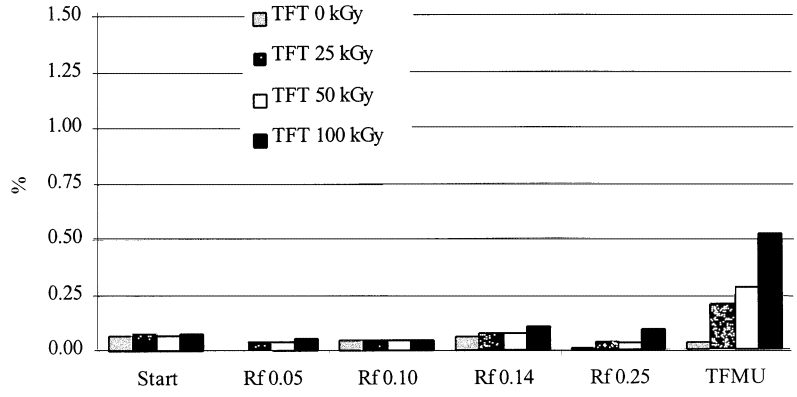

Fig. 9. Impurity profile of TFT after electron-beam irradiation under ambient atmosphere performed densitometrically at a fixed wavelength of 254 nm.

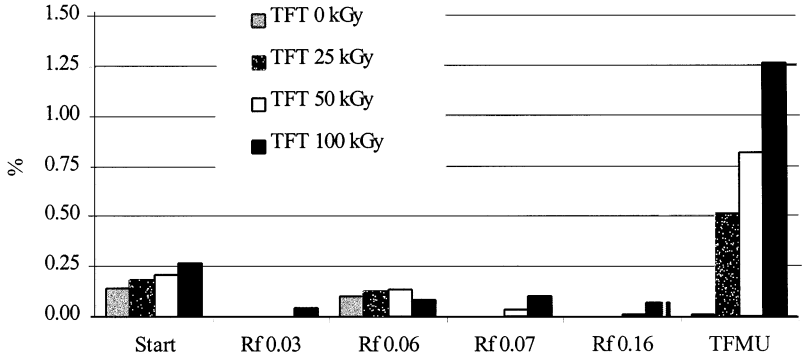

Fig. 10. Impurity profile of TFT after γ-irradiation under ambient atmosphere performed densitometrically at a fixed wavelength of 254 nm.

3.4. Identification of impurities by HPLC-ESI-MS

The major degradation products were identified with HPLC-MS. As stated above apart from TFMU mainly one product generated from radiolysis. So this analysis was performed principally to identify this degradation product RRT 0.68. This product was shown to arise solely by irradiation. The ESI-MS analysis showed a $[M-H]^-$ quasi-molecular ion at m/z 311 (Fig. 13) giving a molecular weight of 312 g/mol. The main fragment ion (m/z 179) corresponded to TFMU (for comparison, see Fig. 14). This means that the trifluoromethyluracil-group was not affected. Consequently the degradation reaction was supposed to take place in the sugar moiety. The probably product is an acyclic compound oxidized in the 4′-position (Fig. 15). Similar reactivity has been observed after irradiation of DNA. Several sugars oxidized in 4′-position were isolated and among others 2-deoxypentose-4-ulose has been identified (Lee, 1987).

Table 1
Limit of detection of 2-deoxyribose (dR)

Reagent (Merck, 1970)	Limit of detection
cysteine hydrochloride reagent	20 ng (UV 366 nm) 100 ng (UV 254 nm)
Na-metaperiodate/p-nitroaniline reagent	>400 ng (VIS/UV 366 nm)
Diaminobenzoic acid reagent	90 ng (UV 366 nm)

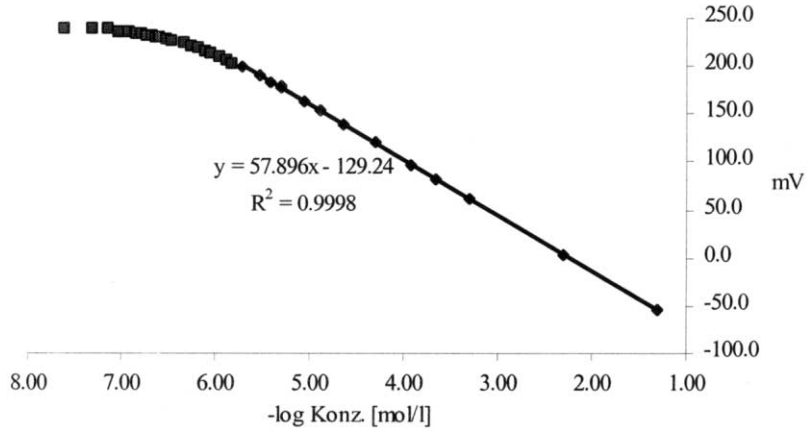

Fig. 11. Calibration curve of free fluoride, measured with a fluoride ion sensitive electrode.

Furthermore four impurities with a molecular weight 412 g/mol (m/z 411) were detected. They were assumed to be reaction products of TFT and dR. The reaction could take place in various positions of dR because radicals may generate in all the positions C1' to C5' (Lee, 1987). The degradation pathway in Fig. 15 shows the combination product of TFT and dR in position 1'.

Another unknown degradation product was detected with a molecular weight 310 g/mol (m/z 309). It might be an oxidation product (–2H) of the degradation product RRT 0.68 (mol. wt. = 312 g/mol).

The results of the LC-MS analysis illustrated that all degradation products still had an unaltered TFMU-unit because the fragment m/z 179 was found in all impurities. Therefore the main reactions were supposed to take place in the sugar moiety. The major degradation product TFMU was detected in irradiated TFT as well (Fig. 14).

3.5. Degradation pathway

Fig. 15 presents a possible degradation pathway of TFT after γ-irradiation. It derives from the identified impurities.

It is supposed that TFMU was formed by two different ways: homolytic cleavage of the glycosidic bond and formation by the intermediate product (312 g/mol) producing 2-deoxypentose-4-ulose.

5-Hydroxydifluoromethyl was not detected in irradiated TFT. But the presence of 5-carboxydeoxyuridine and the increase of free fluoride lead to the conclusion that it existed at least as an intermediate product.

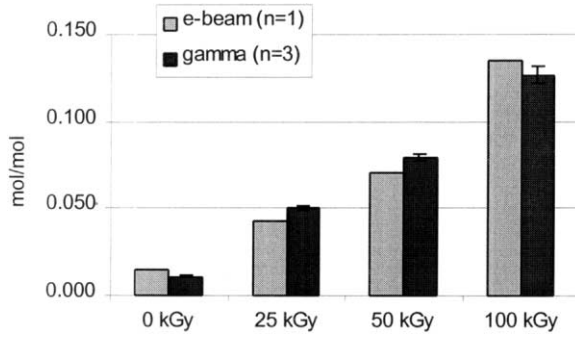

Fig. 12. Concentration % [mol/mol] of fluoride in irradiated TFT measured with a fluoride ion sensitive electrode.

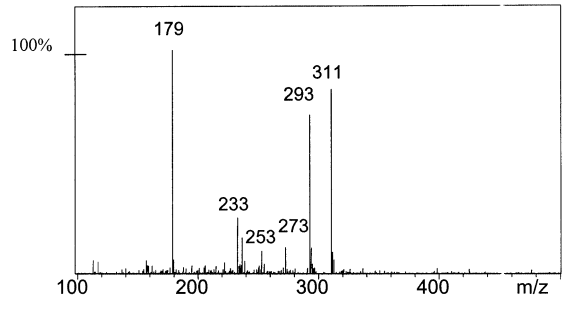

Fig. 13. Negative ESI-MS of the radiolysis product RRT 0.68.

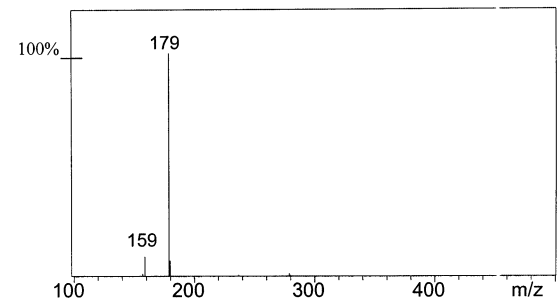

Fig. 14. Negative ESI-MS of TFMU.

5-Carboxyuracil was identified in irradiated TFMU. In irradiated TFT it was not found.

The sugar moiety was degraded during irradiation. It also could have reacted with TFT as it is proposed in Fig. 15. Deoxyribose itself was shown in our studies to be sensitive to ionizing radiation.

Decarboxylation reaction to deoxyuridine and uridine is not assumed according to these results.

4. Conclusion

TFT is reasonably stable to radiation sterilization. The degradation after electron-beam irradiation is significantly lower than after γ-irradiation. At the standard irradiation dose of 25 kGy, the sum of radiation-induced products is about 0.25% (e-beam) and about 0.50% (gamma). Dry heat treatment at 140°C for 8 h showed no significant degradation. But sterilization at 160°C is excluded

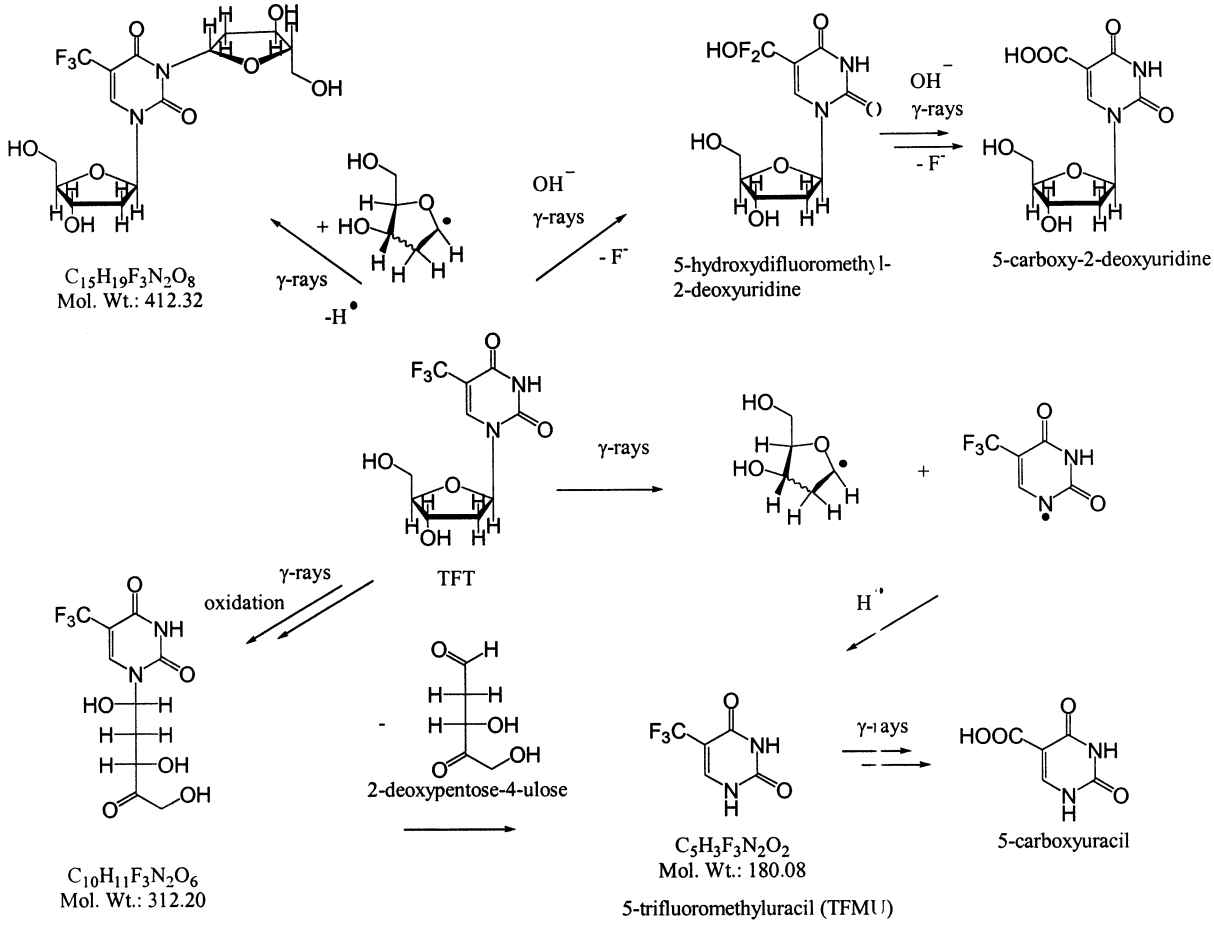

Fig. 15. Degradation path of TFT.

because TFT undergoes an excessive break-down. Therefore sterilization is preferred to be performed with ionizing radiation, if possible with an electron accelerator. Efficacy and toxicity of the irradiated drug substance should be tested with suitable methods, but neither the presence of harmful radiolysis products nor a decrease of the efficacy is assumed.

References

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