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The Influence of Oxygen and Nitrogen on the Radiolysis of Chloramphenicol

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Abstract: This study investigates the influence of oxygen and nitrogen on the radiolysis of chloramphenicol (CAP). The drug substance was irradiated with 25, 50 and 100 kGy (^{60}Co-source) under nitrogen, oxygen and ambient atmosphere, respectively. The content and the impurity profile of CAP were determined with several analytical methods. The content was determined by HPLC as well as by DSC. No significant (p = 95%) difference was found between CAP irradiated under oxygen and nitrogen atmosphere. Purity analysis of CAP was determined by liquid chromatography and by two thin-layer chromatographic systems. Nitrogen was found to act as a scavenger. Several degradation products decreased under nitrogen atmosphere. Oxygen neither increased the sum of impurities significantly nor induced any new radiolytical products. Furthermore, oxygen also showed a scavenging effect. The extent of the radiolysis of CAP (0.5–1.0%) was on an acceptable level. The known impurities 4-nitrobenzaldehyde and 4-nitrobenzoic acid amounted to less than 0.10%. AMPD and two further known impurities were above a threshold of 0.1%.

Keywords: Chloramphenicol · γ-Irradiation · Nitrogen · Oxygen · Pharmaceutical chemistry · Scavenger

1. Introduction

The radiation sterilization of medical devices with γ-rays or with a high-energy electron-beam is an established procedure. In many countries 25 kGy of absorbed radiation is historically selected to be the minimum sterilization dose [1]. This dose must be optimized to reduce the degradation of sensitive products. The optimized radiation dose has to be determined in accordance to the natural radiation resistance of the microbial population of the product to have a probability of a nonsterility level of 1:1000000 [1]. It was shown that medical devices could be sterilized with a sterilization dose of 17.3 kGy even considering a theoretical 'worst case' [2].

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With the concerns about the safety of ethylene oxide, ionizing radiation sterilization should be applied to drug substances and final dosage forms unable to withstand heat sterilization [3]. Generally in new drug substances a threshold limit of 0.1% is defined below which qualification of the impurities is not needed [4]. Therefore analytical methods with high sensitivity and selectivity have to be evaluated. Altorfer *et al.* applied different analytical methods in order to investigate the radiation-induced effects of CAP [5]. Liquid chromatography was found to be the most suitable method due to its high selectivity and sensitivity. Thermometric analysis using differential scanning calorimetry showed good results too provided the substance melts without decomposition and the by-products are soluble in the melted CAP-fractions. In contrast, quantitative thin layer chromatography was shown not to be suitable for assay comparing irradiated and non-irradiated CAP.

Effects of ionizing radiation on CAP have been investigated several times. Fig. 1 shows the structure of CAP. It was found that the degradation of the drug substance was about 0.5–2.0% [5–8]. Solutions of CAP were shown not to withstand γ-irradiation [6][7]. The decomposition pathway is very complex. Radicals such as H·, HO_2·, ·OH and solvated electrons arising from the radiolysis of traces of water can induce many by-products [10]. The influence of residual solvents like methanol or chloroform producing radicals during the irradiation is difficult to determine. Any radicals of CAP can recombine in the solid drug substance. Possible impurities are shown in Fig. 2.

During irradiation nitrogen acts as a scavenger replacing oxygen in the sterilized product [9]. Oxygen also acts as a scavenger, catching the exceedingly reducing solvated electrons (e^-_{sol}) and the hydrogen radicals (H·). Furthermore it increases the radiolysis performing short-lived radical anions and cations such as

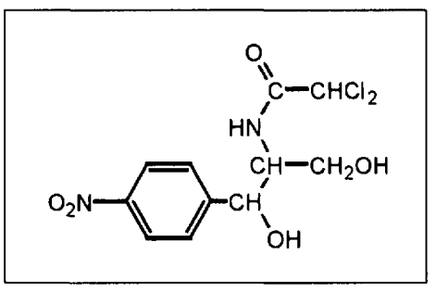

Fig. 1. Structure of CAP

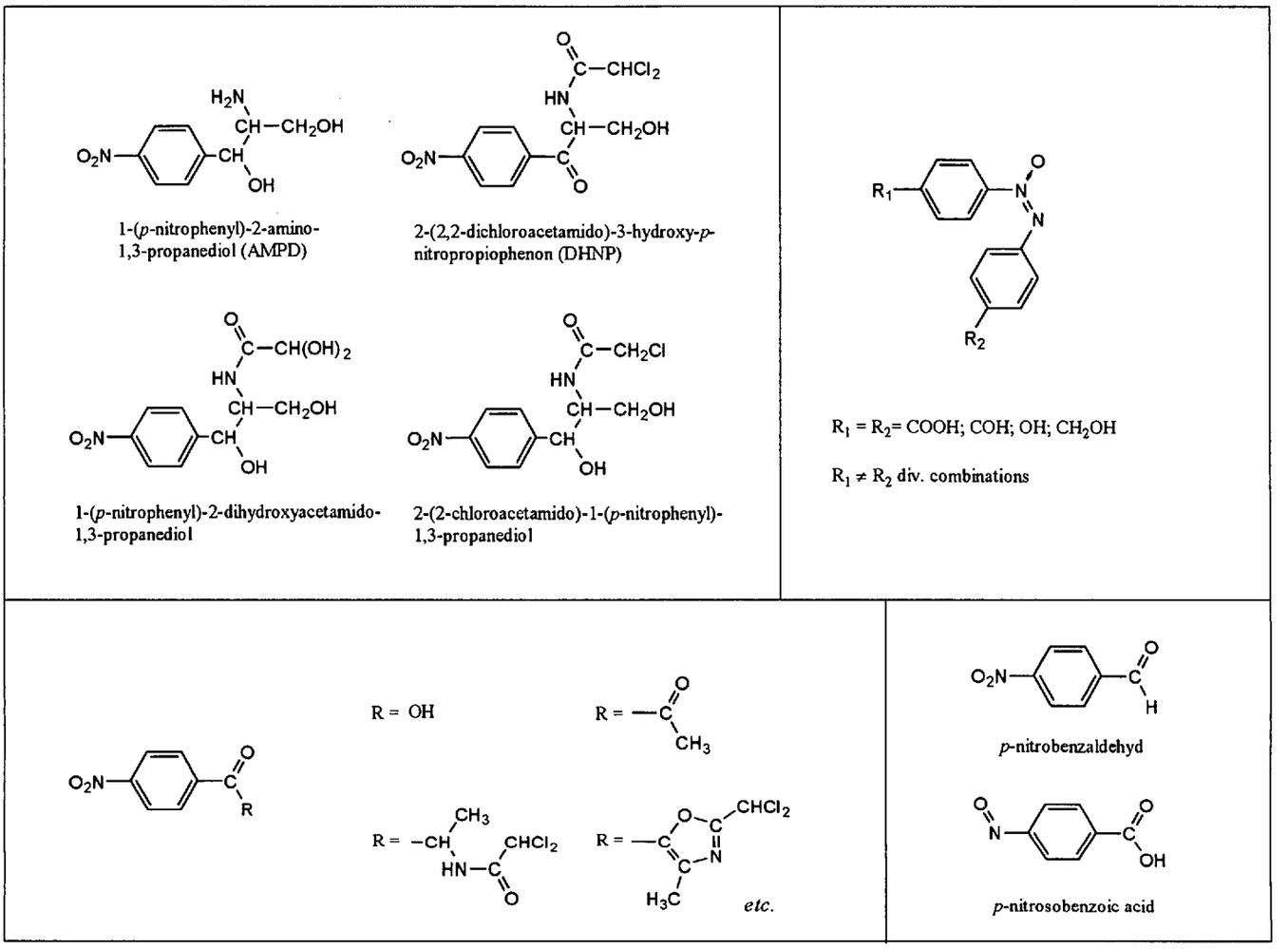

Fig. 2. Possible impurities of CAP after γ-radiation

O_4^+, O_4^-, O_2^+, O_2^-, and O^+ as well as the strongly oxidizing ozone [10]. It is known that oxygen increases the sensitivity to γ-rays of many microorganisms [11]. So irradiation under nitrogen atmosphere could lead to an increase of the effective irradiation dose.

Due to the fact that γ-irradiation is the only possibility to sterilize and store CAP economically, the present work investigates the influence of nitrogen and oxygen on the radiolysis of CAP by simple gazing the drug substance. The results will show whether the degradation is on an acceptable level or if further scavengers have to be tested in order to use γ-irradiation as an effective and economical method of sterilization in the pharmaceutical industry.

2. Experimental

2.1. *Materials and Irradiation*

Chloramphenicol was kindly supplied by the CIBA-Vision company (CH–8442 Hettlingen, Switzerland). For the irradiation the drug substance was purged and packed into polyethylene bottles under nitrogen and oxygen atmosphere, respectively. All other reagents were of adequate analytical grade.

The samples were treated with gamma rays at r.t. from a ^{60}Co (IR-168) (Studer AG 4658 Däniken, Switzerland). The dose rate was 6.9 kGy/h. The non-irradiated CAP was stored as a quasi-standard set to 100%. All samples were correlated to the non-irradiated sample.

2.2. *Content*

2.2.1. *Liquid Chromatography*

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

Stationary phase: Stainless steel column (125 × 4 mm i.d.) packed with LiChrospher RP 18 (particle size 5 µm; Merck KGaA, 64271 Darmstadt, Germany) 'Purospher®'.

Mobile phase: A mixture of water, methanol and glacial acetic acid (55:45:1)

Flow rate: 1.0 ml/min

Injection volume: 10 µl with the cut method (sample loop 100 µl)

Sample preparation: About 15.00 mg of sample were weighed and dissolved with mobile phase in a 100.0 ml volumetric flask.

Calculation: At a fixed wavelength of 280 nm by area.

Statistical tests: Statistical tests were performed with formula F1 and F2 at a decision level of p = 95%

(F1) *F*-Test: $F_{cal} = \frac{s_1^2}{s_2^2}$

(F2) *t*-Test: $t_{cal} = \frac{|x1 - x2|}{Sp}\sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}}$

whereas, $s_p = \sqrt{\frac{(n_1 - 1)\cdot s_1^2 + (n_2 - 1)\cdot s_2^2}{n_1 + n_2 - 2}}$

2.2.2. *Differential Scanning Calorimetry*

Instrumentation: Perkin Elmer differential scanning calorimeter DSC7 (Perkin-Elmer Corporation, Norwalk, CT, 06859 USA).

Start temperature: 100 °C was held for 30 sec.

Scanning rate: 5 °C/min

Final temperature: 180 °C

Sample preparation: About 3.000 mg of homogenized sample was filled in a double-bottom aluminum sample pan.

Reference: An empty double-bottom sample pan was used.

Calculation: According to the slope of the melting curves, a van't Hoff plot was calculated from which the purity data were extrapolated.

Statistical tests: Statistical tests were performed with formula F1 and F2 at a decision level of p = 95%

2.3. *Purity*

2.3.1. *Liquid Chromatography*

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

Stationary phase: Stainless steel column (250 × 4 mm i.d.) packed with LiChrospher 60 RP select B (particle size 5 µm; Merck KGaA, 64271 Darmstadt, Germany).

Mobile phase: Gradient elution consisting of (A) acetonitrile and (B) 20 mM phosphate buffer pH 2.5 as follows: 0 min 20% A – 20 min 50% A – 30 min 70% A.

Flow rate: 1.0 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 the calculations were performed at 278 nm. To calculate the amount of the impurities, the areas were correlated to a CAP-solution that corresponds to 0.5% impurities, without correction of the responses of the known impurities.

2.3.2. *Thin-layer Chromatography*

Stationary phase: 20 × 20 cm TLC plates coated with 0.25 mm silica gel 60 F_{254} (Merck KGaA, 64271 Darmstadt, Germany), prewashed with a mixture of dichloromethane and methanol (80:20) and dried at r.t.

Mobile phase 1: Chloroform:methanol: water 90:10:1 [13]

Mobile phase 2: Chloroform:methanol: glacial acetic acid 79:14:7 [12].

Development path: 160 mm

Chamber: Normal chamber saturated for *ca.* 30 min

Sample application: Aliquots of 20 µl were applied on the plates (automatic application device ATS 3 (Camag, 4132 Muttenz, Switzerland) in 10 tracks of 10 mm width spaced by 6.5 mm using nitrogen as carrier gas. Samples irradiated under the same atmosphere were applied on the same plate.

Sample preparation: About 10.00 mg of sample were dissolved in 1000 µl of methanol.

Calculation: The measurements were performed with a Camag Scanner 3 (Camag, 4132 Muttenz, Switzerland) at a wavelength of 280 nm and data processed using Cats4 chromatography software. The calculations were made by the data-pair method. To calculate the amount of the impurities, the areas were correlated to a CAP-solution that corresponded to 0.5% impurities, without correction of the responses of the known impurities.

3. Results and Discussion

3.1. *Content*

After irradiation the content of CAP decreases significantly. At an absorbed dose of 25 kGy the content is about 99%. The results of the assays are given in Table 1. No significant difference is found

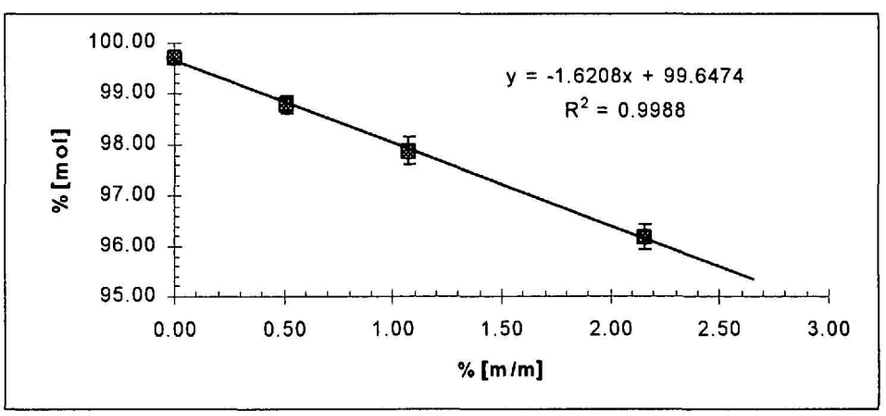

Fig. 3. Relation of DSC-content [mol%] and impurities [weight%] with known impurities 4-NBA, 4-NBS and AMPD (3+3+4) (n=5)

Table 1. CAP irradiated under nitrogen (N_2), oxygen (O_2) and ambient (air) atmosphere, respectively. DSC content is calculated in %(mol/mol) [mol%] and converted into %(m/m) [%].

	HPLC (n = 5, 10[a])		**DSC** (n = 5)		
CAP-sample	content [%]	RSD[b]	[mol %]	[%]	RSD
0 kGy	100	0.60	100	100	0.02
air 25 kGy	98.81	0.78	97.72	98.81	0.05
N_2 25 kGy	99.40	0.46	98.23	99.13	0.05
O_2 25 kGy	98.91	0.82	98.21	99.11	0.06
air 50 kGy	97.75	0.58	96.19	97.87	0.14
N_2 50 kGy	98.39	0.72	97.10	98.43	0.10
O_2 50 kGy	98.21	0.71	96.92	98.30	0.16
air 100 kGy	95.24	1.38			
N_2 100 kGy	95.26	1.27			
O_2 100 kGy	95.84	0.73			

[a]n = 5: for the samples irradiated under nitrogen and oxygen atmosphere
n = 10: for the non-irradiated CAP and the samples irradiated under ambient atmosphere
[b]RSD = relative standard deviation.

Table 2. Sum of impurities [%] of CAP after irradiation under oxygen, (O_2) nitrogen (N_2) and ambient (air) atmosphere, respectively.

	HPLC (n = 3)		TLC mob. phase 1 (n = 6)		TLC mob. phase 2 (n = 6)		DSC[a] (n = 5)	
CAP-sample	%	RSD[b]	%	RSD	%	RSD	%	RSD
air 25 kGy	0.72	1.8	0.61	4.7	0.56	12.5	1.19	1.9
N_2 25 kGy	0.65	0.5	0.63	6.0	0.42	9.6	0.87	2.4
O_2 25 kGy	0.73	1.1	0.65	3.9	0.49	8.3	0.89	2.9
air 50 kGy	1.35	0.9	1.19	3.0	0.81	8.9	2.13	3.4
N_2 50 kGy	1.37	1.4	1.36	8.7	0.86	8.0	1.57	3.1
O_2 50 kGy	1.39	0.5	1.21	3.0	0.91	5.5	1.70	4.7
air 100 kGy	2.62	0.9	2.29	5.5	1.45	10.2		
N_2 100 kGy	2.53	1.6	2.37	6.1	1.55	12.1		
O_2 100 kGy	2.64	2.1	2.33	3.1	1.48	8.4		

[a] DSC: Impurities = 100%-corrected content [%]
[b] RSD = relative standard deviation.

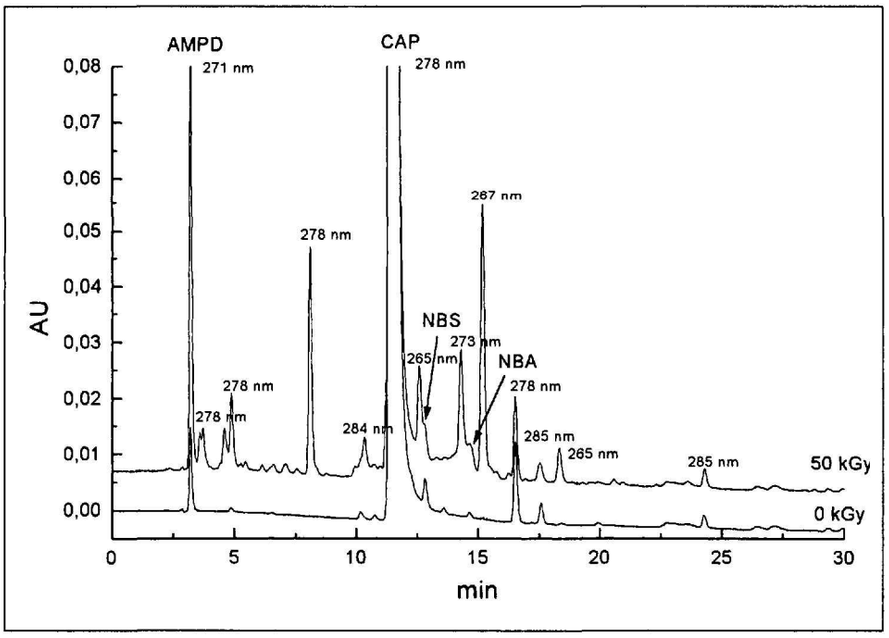

Fig. 4. Chromatograms of non-irradiated and irradiated CAP obtained with the HPLC purity method (λ = 278 nm)

between CAP irradiated under oxygen and under nitrogen atmosphere. According to the content neither nitrogen nor oxygen have a scavenging effect.

The thermometric method has a significantly higher precision than the chromatographic method. Therefore DSC is a very suitable method to determine small differences in content. However, DSC-content is calculated in mol%. So non-irradiated CAP was spiked with known impurities AMPD, NBA and NBS. Fig. 3 illustrates that the content [mol%] is in a linear relation to the amount of impurities [m/m%]. The corrected values are given in Table 1. They tally with the HPLC values.

3.2. *Purity*

3.2.1. *Sum of Impurities*

The results of all methods are summarized in Table 2. It shows that the sum of impurities increases significantly in correlation with the irradiation dose. In contrast, there is no significant difference between the samples irradiated under nitrogen and oxygen atmosphere.

3.2.2. *Impurity Profiles*

3.2.2.1. *HPLC*

Typical HPLC chromatograms of the purity analysis are shown in Fig. 4. After irradiation with 50 kGy many degradation products are detectable. Four impurities with retention times between 5 and 7 min have maximum absorption in a wavelength range from 300 to 320 nm. The extinction maximums of the impurities (>0.05 area%) are given. AMPD, *p*-nitrobenzaldehyde (NBA) and *p*-nitrobenzoic acid (NBS) are identified by HPLC. These three impurities generate from hydrolysis or from UV irradiation as well [14–16]. NBA and NBS are not separated from other impurities. So quantification of these two known impurities is not possible with this system.

The impurity profile of CAP after irradiation with 25 kGy is shown in Fig. 5. In contrast to the sum of impurities, differences between irradiation under nitrogen and oxygen atmosphere are evident. The same differences are determined with 50 and 100 kGy of absorbed dose. Most of the degradation products arise in presence of oxygen in a higher amount including AMPD and the major impurity RRT 1.31. In contrast, two radiolytic products RRT 0.68 and 1.23 are increased after irradiation under nitrogen atmosphere. Thus oxygen has a scavenging effect too. Impurity RRT 0.68 has been identified as a monochloro-compound [17]. Oxygen probably acts as a scavenger suppressing the fission of the carbon-chlorine bond.

Using HPLC 13 impurities (> 0.05 area%) are detected and about eight impurities using the TLC methods. Further radiolytic products (< 0.01 area%) are detectable using HPLC. The sum of known impurities is about 0.19–0.35%. About 0.05–0.12% 4-NBA, 4-NBS and AMPD, respectively, are generated from irradiation with a dose of 25 kGy.

3.2.2.2. *TLC*

Fig. 6 and 7 present the chromatograms obtained with the TLC-systems. Mobile phase 1 is more suitable than mobile phase 2, especially the separation of NBA and NBS is better. In contrast, AMPD is separated with higher resolution with mobile phase 2. So purity analysis was performed with both TLC-systems in order to quantify all known impurities.

Mobile phase 1: Fig. 8 shows the impurity profile of CAP after γ-irradiation with 25 kGy. Seven impurities are separated. With higher irradiation dose three

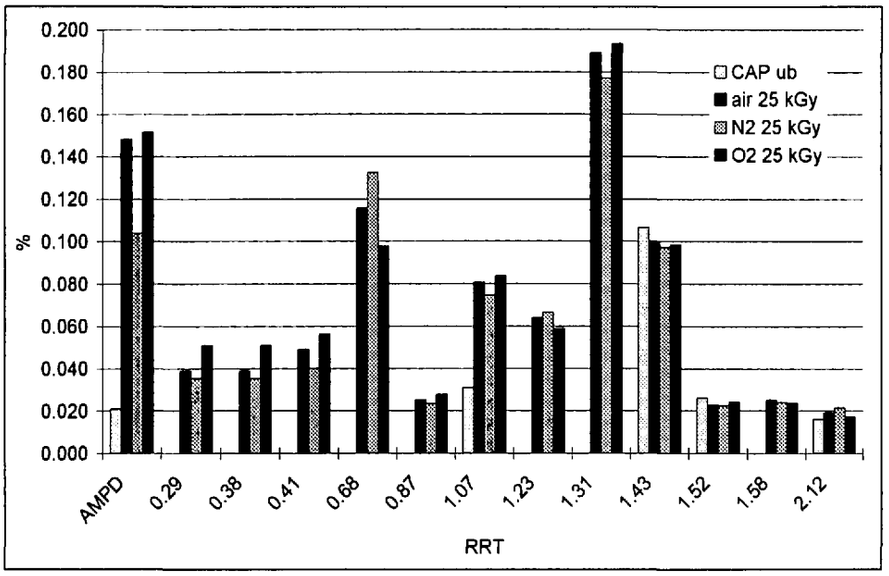

Fig. 5. Impurity profile of CAP after irradiation with 25 kGy under nitrogen (N_2), oxygen (O_2) and ambient (air) atmosphere, respectively. RRT = relative retention time to CAP

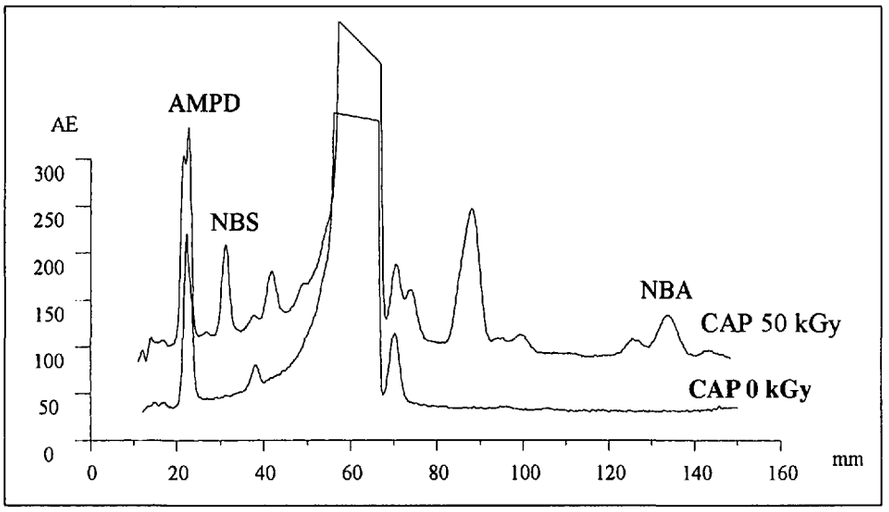

Fig. 6. Chromatograms of non-irradiated and irradiated CAP obtained with the mobile phase 1 (λ = 278 nm)

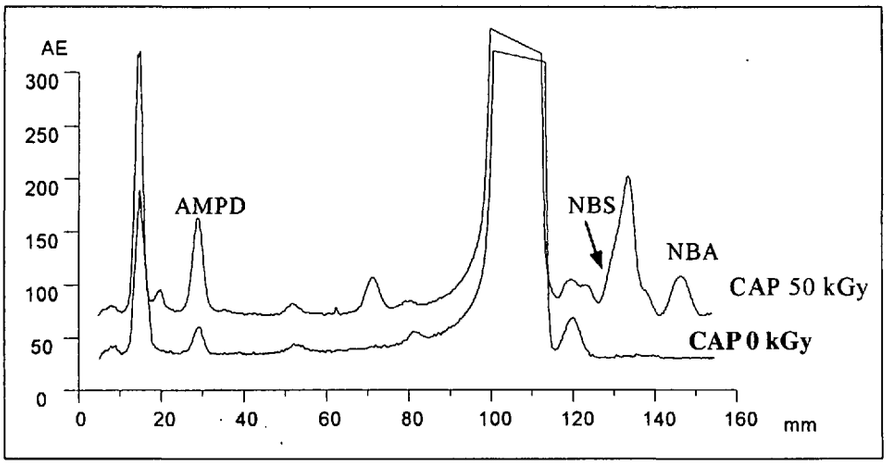

Fig. 7. Chromatograms of non-irradiated and irradiated CAP obtained with the mobile phase 2 (λ = 280 nm)

additional degradation products Rf 0.17, 0.49 and 0.63 are detectable. There are small differences between the irradiation under oxygen and nitrogen atmosphere. Five impurities show an increase in the presence of oxygen. Impurity Rf 0.40 arises in a higher amount after irradiation under nitrogen atmosphere. This impurity corresponds to impurity RRT 0.68 of the HPLC analysis.

Mobile phase 2: Totally seven impurities are detectable with this TLC system. All of them arise in the smallest amount when CAP has been irradiated under nitrogen atmosphere, as shown in Fig. 9. The scavenging effect of oxygen is not detectable with this TLC system.

4. Conclusions

Neither nitrogen nor oxygen show a significant influence on the sum of impurities after γ-irradiation of CAP. The same degradation products were generated in different amounts. According to the purity analysis, both nitrogen and oxygen have a scavenging effect. So the irradiation should be performed under normal atmosphere in presence of oxygen in order to avoid increasing the radiation resistance of the microorganisms.

The sum of all results leads to the conclusion that γ-radiation sterilization presents itself for the manufacture of sterile CAP under normal atmosphere. The degradation is only about 0.5–1.0% with 25 kGy of absorbed dose. Due to the fact that the extent of the radiolysis depends much on the absorbed dose, the sterilization dose should be optimized. But priority must be given to the investigations of the effectiveness and toxicity of the irradiated drug substance.

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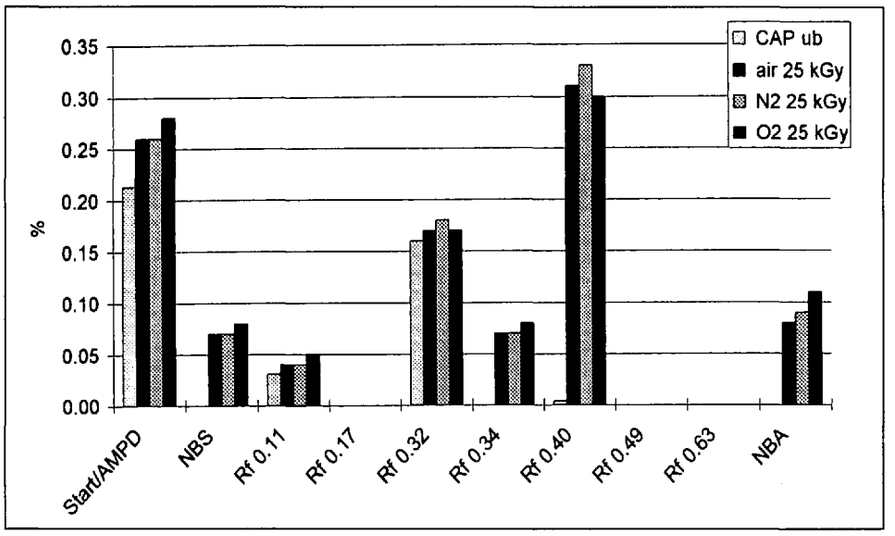

Fig. 8. Impurity profile of CAP after irradiation with 25 kGy under nitrogen (N_2), oxygen (O_2) and ambient (air) atmosphere, respectively. (TLC mobile phase 1)

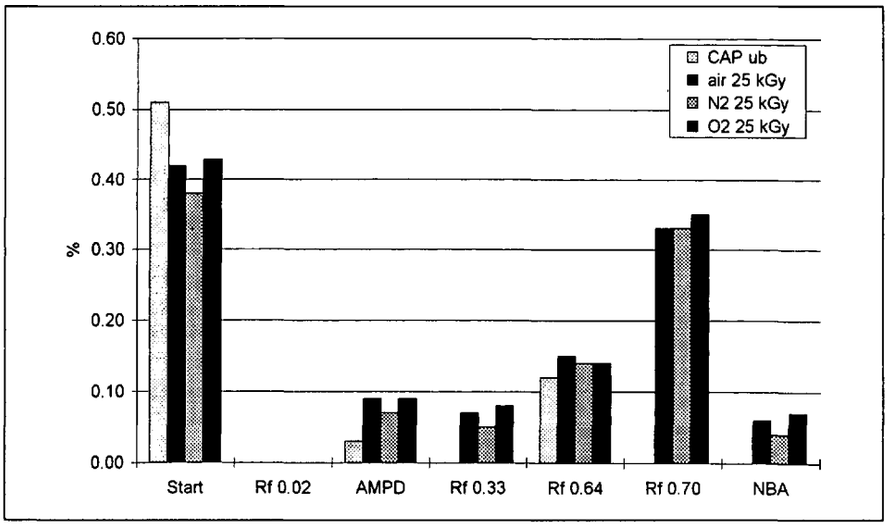

Fig. 9. Impurity profile of CAP after irradiation with 25 kGy under nitrogen (N_2), oxygen (O_2) and ambient (air) atmosphere, respectively. (TLC mobile phase 2)

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