Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 343 (2007) 4–11
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Irradiation of human insulin in aqueous solution, first step towards radiosterilization

Hélène Terryn [a], Aubert Maquille [a,*], Chantal Houée-Levin [b], Bernard Tilquin [a]

[a] *Laboratory of Chemical and Physicochemical Analysis of Drugs (CHAM), Université Catholique de Louvain, Brussels, Belgium*
[b] *Team of Radiation Biochemistry, UMR 8000 CNRS—université Paris XI, Orsay, France*

Received 22 January 2007; received in revised form 5 April 2007; accepted 8 April 2007
Available online 24 April 2007

Abstract

The degradation of irradiated human insulin in aqueous solutions was investigated in order to protect the protein against ionizing radiation. The influence of the drug concentration, excipients and irradiation temperature were studied.

Aqueous solutions at pH 2 were irradiated by gamma rays or by accelerated electrons. Two different high-performance liquid chromatography (HPLC) methods were used: reverse-phase high-performance liquid chromatography (RP-HPLC)/UV and size exclusion liquid chromatography (SEC/UV) to investigate both the fragmentation and the formation of higher molecular weight proteins.

In solution without excipients irradiated at ambient temperature at 10 kGy, the loss of human insulin is almost complete. Addition of radio-protecting excipients (free radicals scavengers) and cryo-irradiation allowed to decrease insulin degradation. The best radio-protector used was ascorbic acid in aqueous solution and oxidized glutathione in the frozen solutions. Only the combination of these two approaches (addition of scavenger and freezing) enables the irradiated human insulin in aqueous solution to meet the European Pharmacopoeia requirements for chemical potency ($\geq$90%).

Keywords: Radiolysis; Human insulin; Aqueous solution; HPLC

1. Introduction

Human insulin (HIns) is a hypoglycemic protein hormone. Usually, it is administered through intravenous or subcutaneous injection and thus has to meet the Pharmacopoeia requirements of sterility. These insulin preparations are sterilized by aseptic filtration because of their heat sensitivity. Aseptic processing is costly and does not lead to a 10^{-6} sterility assurance level; furthermore, pharmaceutical guidelines recommend terminal sterilization methods (European Pharmacopoeia, 2005; EMEA, 2000; USP, 2000). Sterilization by ionizing radiation could be a good alternative since it can be applied on the final packaged product and gives no rise in temperature (Reid, 1995; Nordhauser and Olson, 1998). Radiation processing techniques have evolved so that radiosterilization has become the first choice method for thermosensitive solid state drugs as described in the EMEA decision tree for the selection of sterilization methods (EMEA, 2000). However, the use of ionizing radiation for drugs in aqueous solution is not even considered. There is a consensus that radiosterilization should not be applied to drugs in aqueous solution because of the greater degradation (loss of chemical potency) of the drug compared to the solid state (Tilquin, 1991; Gopal, 1995; Boess and Bögl, 1996; Tilquin and Rollmann, 1996). The degradation of a drug solute in aqueous solution is brought about by the attack of free radicals generated by the water radiolysis and depends on several parameters such as the absorbed dose, the temperature and the drug concentration.

The purpose of the present work is to quantify the degradation of human insulin upon irradiation as a contribution to a final sterilization technique. Two methods were investigated to lower the degradation of the drug solute:

- The use of radio-protecting excipients. As the degradation of the drug solute results from the indirect effect through reactions with reactive species of water radiolysis, scavengers of these reactive species could protect the drug solute. The choice of the scavenger and its concentration will depend

* Corresponding author. Tel.: +32 2 764 72 97; fax: +32 2 764 72 96.
E-mail address: aubert.maquille@cham.ucl.ac.be (A. Maquille).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.04.012

on the solute concentration and reactivity with the radicals responsible for the solute degradation.
- Cryo-irradiation. The temperature is lowered by freezing the drug solution which limits the diffusion of reactive species formed (indirect effect).

This work was conducted at pH 2 because of the very low solubility of insulin at neutral pH. Insulin is very weakly soluble in neutral medium and in addition, it precipitated under irradiation, mostly because of the opening of the disulfide bonds. Radiolysis in neutral solution is less oxidant and the three disulfide bonds are sensitive to reduction.

The residual human insulin was measured by high-performance liquid chromatography (HPLC)-UV and the influence of the absorbed dose, the temperature and the radioprotection by excipients were assessed.

2. Materials and methods

2.1. Materials

Neat human insulin (insulin USP, human recombinant, crystalline, residual moisture 7.5%, zinc content 0.42%) was purchased from Celliance^{TM} Corporation (a Serologicals Company) and stored in the dark at 253 K to ensure stability. It was dissolved in 0.05 M phosphoric buffer (pH 2) to obtain concentrations of 10^{-4}, 5×10^{-4} and 10^{-3} M.

Tridistilled water or water coming from an Elga Maxima device was used to prepare all solutions. Several pharmaceutical excipients were added to 10^{-4} M human insulin solutions either those present in commercial insulin injectables such as glycerol, *m*-cresol, methylparaben (methyl 4-hydroxybenzoate), phenol or others such as mannitol, ascorbic acid, oxidized glutathione (GSSG) and reduced glutathione (GSH). Various excipient concentrations were used and are given in Table 1.

D-Mannitol (USP specifications) was purchased from Sigma. Glycerol (extra pure) and L(+)-ascorbic acid (extra pure) were supplied by VWR. Methyl 4-hydroxybenzoate (99%), phenol (99%) and *m*-cresol (USP XXIII) were from Acros Organics.

Six milliliters of samples were put in each vials and protected from light. High-purity nitrogen, supplied by Air-Liquide, was used to saturate the solutions prior to irradiation for 1 h without agitation. The samples were irradiated in their initial solutions without any further pH modification.

Table 1
The concentration (M and % of the different additives tested)

Additives	Concentration (M)	Concentration (%)
Ascorbic acid	3.4×10^{-1}	5.94
Mannitol	2.8×10^{-1}	5.07
Glycerol	2.8×10^{-1}	2.6
Phenol	3×10^{-2}	0.28
m-Cresol	2×10^{-2}	0.22
GSSG	1×10^{-2}	0.6
GSH	1×10^{-2}	0.3
Methyl paraben	6×10^{-3}	0.1

For the cryo-irradiation, the samples were frozen and irradiated in dry ice (198 K) by high-energy electrons. Irradiation of frozen samples could not be performed with gamma rays as, given the time needed to achieve the irradiation, freezing could not be maintained during the whole process. The samples were kept at 253 K after irradiation and were warmed at room temperature just before analysis.

2.2. Gamma and electron-beam irradiations

The HIns aqueous samples were irradiated at ambient temperature protected from the light in a panoramic ^{60}Co chamber (UCL–Louvain–La-Neuve, Belgium) calibrated by alanine dosimetry. The alanine pellets were supplied and analyzed by the Risø National Laboratory in Denmark. The dose rate was 247 Gy h^{-1}. The absorbed doses were 100 Gy, 200 Gy, 500 Gy, 1 kGy, 5 kGy, 10 kGy and 25 kGy. In the figures, only the results obtained for some doses are displayed.

A double beam linear electron accelerator (LINAC) was used for the electron-beam irradiations (Mölnlycke Beta Plant, Waremme, Belgium). The solutions were either irradiated at room temperature or frozen with dry ice. The beam power for each electron generator is 20 kW. The electrons were delivered in pulses of 474 and 478 Hz. The average dose rate was calculated as 3.2×10^7 Gy h^{-1} for the single electron beam and 6.3×10^7 Gy h^{-1} for the double electron beam. A single beam was used to obtain the lowest irradiation dose possible ca. 11 kGy. For higher doses, both beams were used. The samples were irradiated at 11, 16, 24 and 29 kGy. A polymethylmethacrylate (PMM) film was used to control the doses delivered to aqueous solutions and frozen solutions. The absorbed doses were checked with ceric sulfate dosimeters (Ferradini and Pucheault, 1983; Spinks and Woods, 1990) irradiated at room temperature.

2.3. Analytical HPLC methods

The HPLC system was composed of two Kontron 422 HPLC pumps, a Rheodyne® manual injector with a 20 μl sample loop, a Kontron 332 HPLC ultraviolet detector and a Merck Hitachi F-1050 fluorimetric detector. The Borwin® software version 1.5 was used for data acquisition. All the analyses were carried out at room temperature. Calibration curves were constructed for the quantification of human insulin. The formation of dityrosine was monitored by measuring fluorescence after excitation at 325 nm and detection at 410 nm, which ensured selectivity for the detection of dityrosine (Malencik et al., 1996). For the detection of tyrosine, excitation and emission wavelengths were 275 and 300 nm, respectively.

2.3.1. Reverse-phase high-performance liquid chromatography (RP-HPLC) method

A Symmetry300^{TM} C_4 column, 5 μm particle size, 150 × 3.9 mm I.D. (Waters assoc., Milford, Ma, USA) was used. The mobile phase consisted of two eluents: eluent A was 25/75 acetonitrile/water with 0.05% trifluoroacetic acid (TFA) and eluent B was 40/60 acetonitrile/water with 0.05% TFA. A linear gradient from 0% eluent B to 70% eluent B was applied over

40 min. The flow rate was 1 mL/min, and the UV detection wavelength was 214 nm (Linde and Welinder, 1991; Welinder and Sørensen, 1991; Purcell et al., 1995).

2.3.2. Size exclusion chromatography (SEC) method

The SEC method proposed by Brange et al. (1992) was used. Chromatographic separation was performed on a Waters Insulin HMWP (high molecular weight proteins) column, 300 × 7.8 mm I.D. (Waters assoc. Milford, Ma), using 8/92 acetonitrile/water with 2.5 M acetic acid and 4 mM L-arginine at a flow rate of 0.7 ml/min as the mobile phase. The UV detection wavelength was set at 276 nm.

3. Results

3.1. Degradation of insulin

Fig. 1 shows the RP- (Fig. 1A) and SEC-HPLC (Fig. 1B) chromatograms of human insulin 10^{-4} M unirradiated and irradiated at several doses. The inset represents the residual percentage of HIns versus the absorbed dose (kGy).

3.1.1. RP-HPLC

The loss of HIns increased with the absorbed dose. Contrasting with the solid-state irradiation (Terryn et al., 2006), the main impurity present before irradiation (a small peak of A21 desamido-human insulin with a retention time ∼17 min) decreased in aqueous solution as a function of absorbed dose.

3.1.2. SEC-HPLC

At low irradiation doses (from 100 to 500 Gy), the dimer peak (retention time (t_R) = ca.12.5 min) increased with the irradiation dose and a peak of higher molecular mass appeared (t_R = ca.10.9 min). As the irradiation dose increased, the dimer concentration decreased to give way to polymer. For increased irradiation doses, the polymer had lower retention times (t_R for 1 kGy = ca.11.1 min and for 5 kGy = ca.9.9 min), indicating an increase in molecular weight. As seen in Fig. 2A, the fluorescence detection (λ excitation 325 nm, λ emission 410 nm) indicated that dityrosines were present in dimers and polymers. At low irradiation doses (200 Gy to 1 kGy), such fluorescence appeared in the monomer, the dimer and the polymers. On the other hand, at higher doses, peaks of dimer and monomer decreased to give way to polymers of lower retention time and

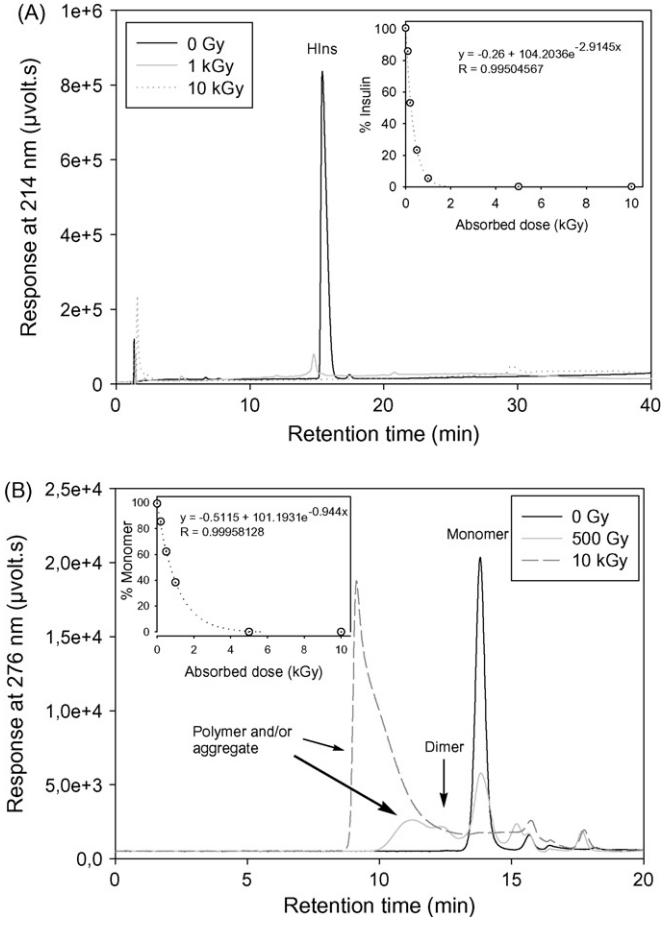

Fig. 1. Overlay of the UV chromatograms of non-irradiated and γ-irradiated human insulin aqueous solutions. HIns 10^{-4} M, nitrogen saturated solution at pH 2. Inset is the percent of non-modified insulin as a function of absorbed dose (kGy). (A) RP-HPLC. (B) SEC-HPLC.

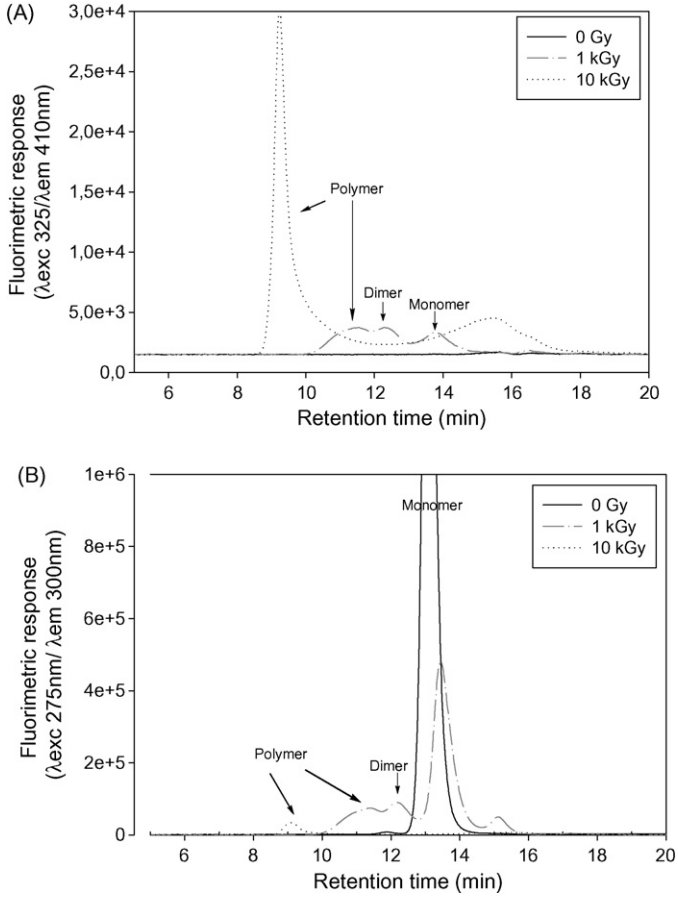

Fig. 2. SEC analysis by fluorescence: overlay of the SEC fluorescence chromatograms of non-irradiated and gamma-irradiated human insulin. HIns 10^{-4} M at pH 2, nitrogen saturated solution. (A) Detection of dityrosine (excitation wavelength 325 nm/emission wavelength 410 nm). (B) Detection of tyrosine (excitation wavelength 275 nm/emission wavelength 300 nm).

another peak with higher retention time. This means that at doses higher than 1 kGy, this dimer underwent radiolysis.

Fluorescence detection at $\lambda_{ex} = 275$ nm and $\lambda_{em} = 300$ nm was used to monitor the presence of tyrosines (Fig. 2B). Whereas tyrosines are detected in dimer and polymer peaks at low irradiation doses (1 kGy), fluorescence due to tyrosines is no longer detected at 10 kGy, except a small peak with retention time of 9.1 min.

The loss of insulin for both RP-HPLC and SEC results followed an exponential law.

3.2. Effect of HIns concentration

Fig. 3 shows the percentage of non-modified HIns measured by RP-HPLC/UV (Fig. 3A) and by SEC/UV (Fig. 3B) as a function of the absorbed dose for gamma irradiations. Three HIns concentrations, within the range of the commercial insulin solutions (100 I.U. corresponds to ca. 6.10^{-4} M), were assayed at pH 2. For the three concentrations, the loss of insulin followed an exponential decay for both RP-HPLC and SEC results.

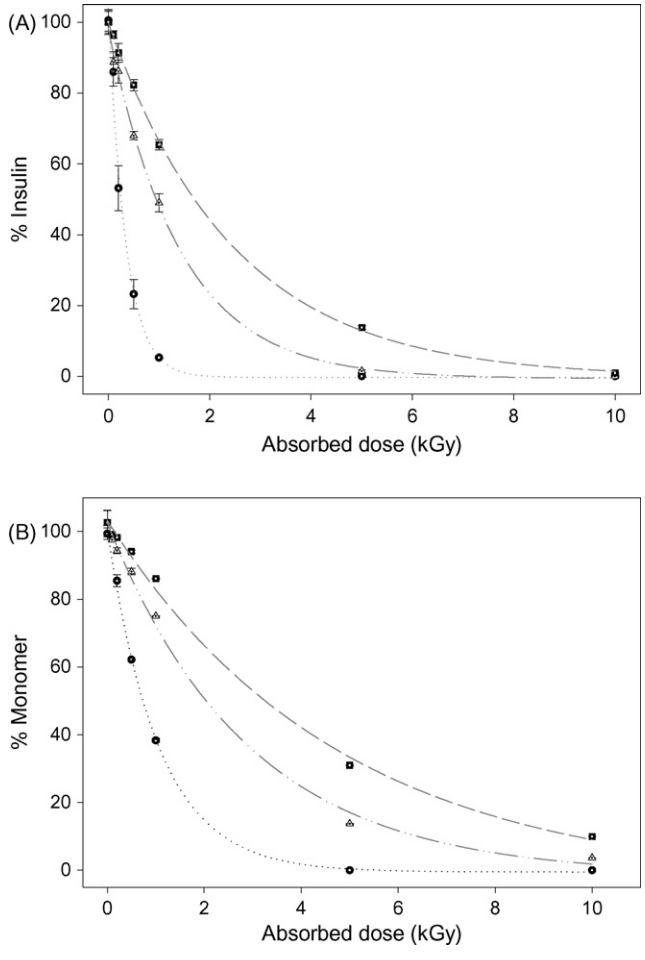

Fig. 3. Variation of the percentage of human insulin (RP-HPLC) or monomer (SEC) as a function of the absorbed dose (kGy) for gamma irradiations for three human insulin initial concentrations. HIns 10^{-4}, 5×10^{-4} and 10^{-3} M at pH 2, nitrogen saturated solutions. A. Results for RP-HPLC/UV B. Results for SEC/UV. (●) Insulin 10^{-4} M; (···) exponential decay for insulin 10^{-4} M; (△) Insulin 5×10^{-4} M; (– ··–) exponential decay for insulin 5×10^{-4} M; (■) Insulin 10^{-3} M; and (– – –) exponential decay for insulin 10^{-3} M.

3.3. Influence of excipients

A HIns concentration of 10^{-4} M was chosen to study the influence of the excipients. The excipients concentrations are reported in Table 1. The concentrations used corresponded either to the maximum percentage allowed in injectables (*m*-cresol, methylparaben, phenol), or to the concentration to get isotonicity to plasma (glycerol, mannitol, ascorbic acid). Oxidized glutathione (GSSG) and reduced glutathione (GSH), which play a role against oxidative stress, were assessed. Since they are not excipients, low concentrations were used. As for radiolysis results, interpretation of the results might be based on free radical scavenging, for which the concentration is the relevant parameter. Thus, for comparison of the effects, the excipients were classified in two groups, the "low concentration" ones (ca. 10^{-2} M) and the "high concentration" ones (ca. 10^{-1} M).

Fig. 4 shows the influence of different excipients on the gamma radiolysis of nitrogen saturated solutions of HIns 10^{-4} M, measured by RP-HPLC/UV.

Concerning the fragmentation (RP-HPLC), in the "low concentration ranges" (Fig. 4A) (*m*-cresol, phenol, methylparaben, GSH and GSSG), GSSG and *m*-cresol were the best radioprotectors followed by phenol, GSH and in the last position methylparaben. As for the "high concentrations" (Fig. 4B)

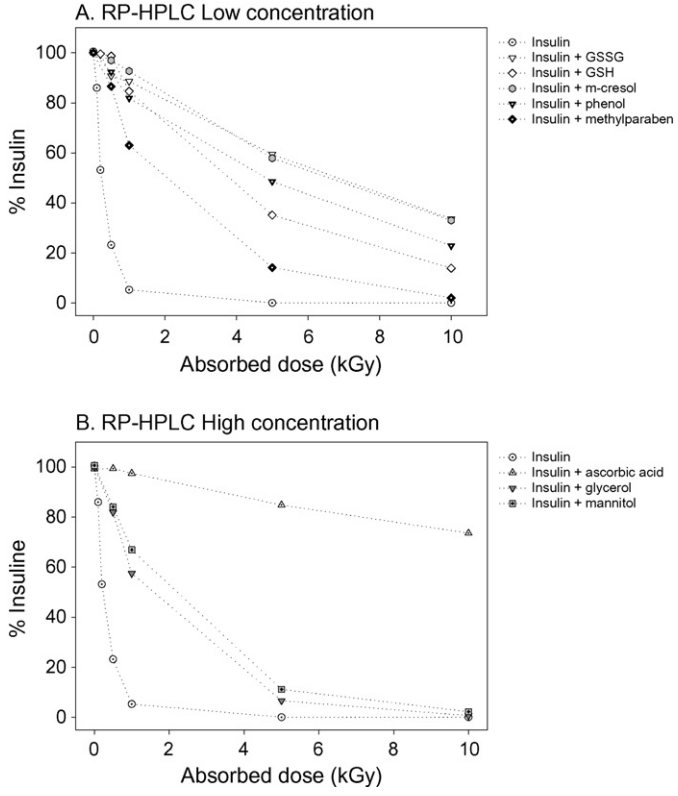

Fig. 4. Influence of excipients on HIns degradation induced by gamma irradiation. HIns 10^{-4} M at pH 2, nitrogen saturated solution. Variation of the percentage of HIns as a function of the absorbed dose. (A) Low concentration. (B) High concentration.

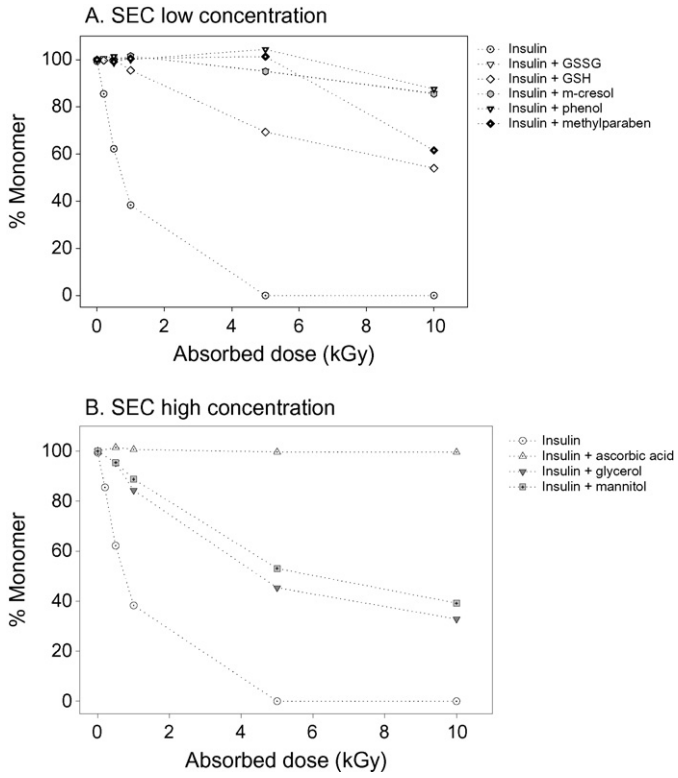

Fig. 5. Influence of excipients on insulin fragmentation induced by gamma irradiation. Percentage of monomer as a function of the absorbed dose. HIns 10^{-4} M at pH 2, nitrogen saturated solution. (A) Low concentration. (B) High concentration.

(ascorbic acid, mannitol and glycerol), ascorbic acid was a very efficient radio-protector (at 10 kGy: 73.6% of non-modified HIns in the presence of 5.94% of ascorbic acid) whilst glycerol and mannitol did not protect HIns to the same extent.

The protective order of the excipients is similar for polymerization (SEC/UV; Fig. 5A and B). Moreover, a greater protection against polymerization than against fragmentation was observed. The protection of ascorbic acid against polymerization was almost complete (99.6% of monomer at 10 kGy).

Since it is the most powerful protecting agent, we focused on ascorbic acid. The effect of the concentration of ascorbic acid on the protection of human insulin against ionizing rays was studied (Fig. 6). For RP-HPLC, the percentage of human insulin remaining after irradiation (10 kGy) increased with ascorbic acid concentration. The protection against the fragmentation depended on the excipient concentration. On the other hand, SEC indicated that for as low as 1% of ascorbic acid, ca. 87% of monomer was still present after irradiation at 10 kGy.

3.4. Influence of the temperature (cryo-irradiation)

Lowering the temperature significantly decreased the degradation of human insulin by ionizing rays. After irradiation at 11 kGy by high energy electrons, the content in non-modified HIns was $84.5 \pm 6.3\%$ in frozen solutions versus $0.23 \pm 0.11\%$ in liquid aqueous solutions.

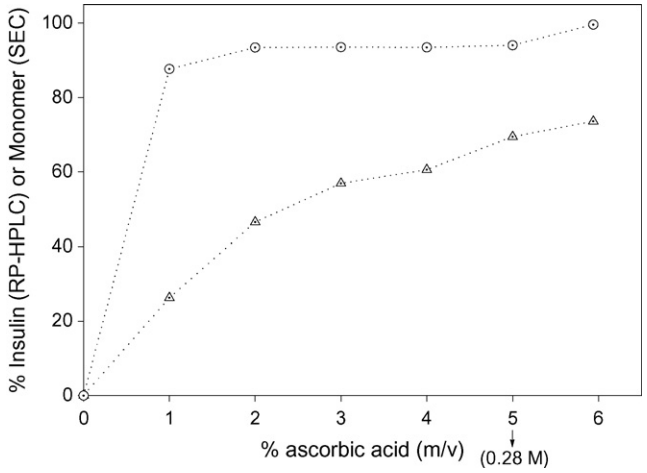

Fig. 6. Variation of the percentage of HIns (for RP-HPLC/UV) or monomer (for SEC/UV) as function of percentage of ascorbic acid (m/v) with gamma irradiation at 10 kGy. HIns 10^{-4} M at pH 2, nitrogen saturated solution. (···△···) RP-HPLC/UV results, (· · · ◯ · · ·) SEC/UV results.

To improve the protection, ascorbic acid (3.4×10^{-1} M) or glutathione (oxidized 10^{-2} M or reduced 10^{-2} M) were added to the solutions. Fig. 7 shows the percentage of non-modified HIns in frozen solutions with and without excipients as a function of the absorbed dose. In both HPLC conditions, the addition of excipients allowed either a decrease or a total disappearance of the degradation peaks of HIns.

4. Discussion

The aim of this study was not to establish a ready-to-use radiosterilization protocol but to make a fundamental study that may help to design such protocol. Solutions were irradiated at pH 2, which does not comply with the requirements for injectable drugs (European Pharmacopoeia, 2005; USP, 2000). However, at this pH value, the protein integrity is maintained and we did not have to face the problem of aggregation.

In diluted liquid solutions at pH 2, irradiation of HIns produces cleavage, dimerization, and polymerizations of the peptidic chains. The fluorescence results indicate that at least some dimers and polymers are linked by dityrosines, which are due to an oxidative process.

In the range of insulin concentration investigated which is within that of commercialized solutions (from 10^{-4} to 10^{-3} M), the radiation chemical yield of HIns is proportional to its concentration. The prognostic of the degradation in this concentration range is possible.

At 10 kGy, which might be considered as a possible radiosterilization dose, human insulin in aqueous solution (in this range of insulin concentration) is destroyed.

Protein damages due to indirect irradiation effects could be minimized by optimization of the irradiation parameters. One approach to achieve this goal is the addition of compounds that act as radioprotectors. Another possibility is to limit the diffusion of reactive species by decreasing the irradiation temperature (Grieb et al., 2002; Amareld et al., 2003; Zbikowska et al., 2006).

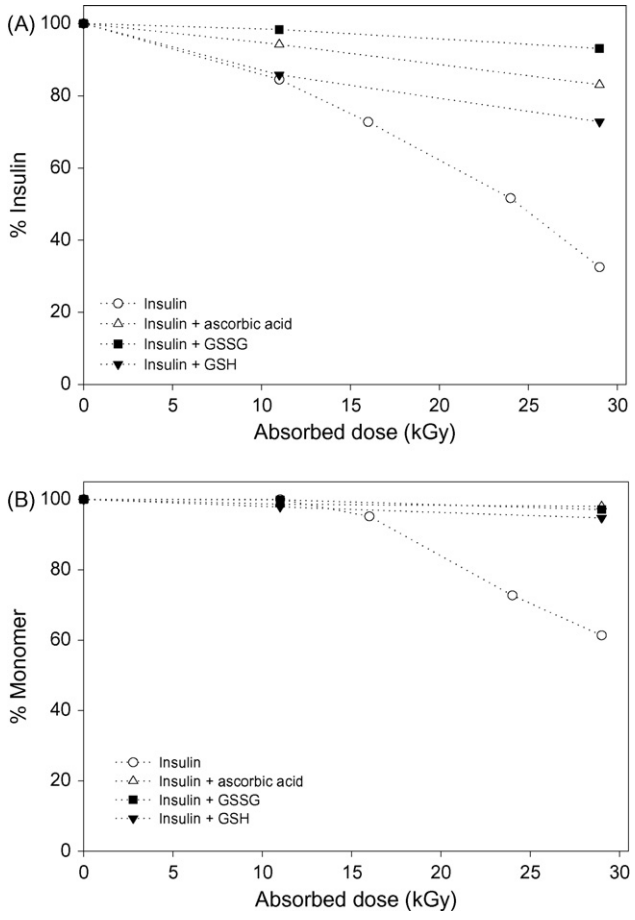

Fig. 7. Percent of non-modified HIns (RP-HPLC/UV) or monomer (SEC/UV) versus absorbed dose (kGy) with electron-beam irradiations in frozen solution with and without excipients. HIns 10^{-4} M at pH 2, nitrogen saturated solution. (···◯···) Insulin, (···△···) Insulin with ascorbic acid 3.4×10^{-1} M, (···■···) Insulin with GSSG 10^{-2} M and (···▼···) Insulin with GSH 10^{-2} M. (A) RP-HPLC/UV results. (B) SEC/UV results.

4.1. Influence of excipients

The radioprotective effect of several excipients was assessed. The bacteriostatic agents (*m*-cresol, phenol, methylparaben) and the isotonic agent (glycerol) are used in insulin preparation. Mannitol is a pharmaceutical excipient that is commonly used in tablets and lyophilized drugs, as a carrier in dry powder and can be administered parenterally (Rowe et al., 2003). Ascorbic acid is a pharmaceutical excipient that is used as antioxidant agent. Oxidized and reduced glutathione are peptides and are not used as pharmaceutical excipients. As they play a role in defense against oxidative stress, one can expect that they have good scavenging capacities against free radicals. Oxidized glutathione has a disulfide bridge.

Ascorbic acid is efficient against degradation and polymerization, but its protective effect against degradation depends on its concentration whereas it is effective against polymerization even at low concentrations (Fig. 6). Degradation and polymerization rely on different mechanisms and different radioprotection mechanisms are involved. The significant decrease of HIns degradation after irradiation in the presence of ascorbic acid seems to be a very promising finding although insulin content after irradiation of a solution containing 5.94% ascorbic acid is still lower than 90%, which is the requirement from the European Pharmacopoeia for injectable preparations of insulin (European Pharmacopoeia, 2005).

Oxidized glutathione is a good radioprotector especially against polymerization. Its disulfide bridge seems to play a role in the radioprotection.

To optimize the search for radioprotectors, it is necessary to have an insight into the mechanisms of radioprotection. In a first step, we focused on the scavenging of water free radicals. The effect of radioprotectors may be simplified as follows:

$$\text{HIns} + \text{free radical} \rightarrow \text{modified protein}$$

$$\text{HIns} + \text{Scavenger} + \text{free radical} \rightarrow \text{unmodified protein}$$

Table 2 gives the absorbed dose that leads to a 10% drug degradation (D10) and the rate constants of reaction of $^{\bullet}$OH with the different excipients used, multiplied by their concentration (the scavenging capacity). The reaction with the aqueous electron at pH 2 is not considered since at that pH, it reacts with the proton. The rate constants of reactions of water free radicals with human insulin are not known, however the values in the database show that the rate constants are of the same order of magnitude

Table 2
D10 (−10%) and rate constants for the reactions of water free radicals with the excipients, multiplied by their concentrations (scavenging capacity)

pH	Type	Hlns concentration (M)	Excipient (M)	D10 (kGy) (RP-HPLC)	D10(kGy) (SEC)	k ($^{\bullet}$OH) (excipient)	k ($^{\bullet}$H) (excipient)
2	γ	10^{-4}	–	0.05	0.12	4.9×10^{6}	5.0×10^{5}
2	γ	5×10^{-4}	–	0.12	0.38	2.5×10^{6}	–
2	γ	10^{-3}	–	0.26	0.63	4.9×10^{7}	–
2	γ	10^{-4}	[HAsc] = 3.34×10^{-1}	3.27	338.41	2.7×10^{9}	5.4×10^{7}
–	–	–	[Asc] = 2.66×10^{-5}	–	–	8.8×10^{4}	–
2	γ	10^{-4}	GSSG 10^{-2}	0.92	7.72	9.3×10^{7}	1.0×10^{8}
2	γ	10^{-4}	GSH 10^{-2}M	0.7	–	2.3×10^{8}	–
2	γ	10^{-4}	*m*-Cresol 2×10^{-2}	1.13	6.75	2.4×10^{8}	–
2	γ	10^{-4}	Phenol 3×10^{-2}	0.61	11.1	2.0×10^{8}	5.1×10^{7}
2	γ	10^{-4}	Methylparaben 6×10^{-3}	0.28	3.99	–	–
2	γ	10^{-4}	Glycerol 2.8×10^{-1}	0.23	0.65	5.6×10^{8}	7.3×10^{6}
2	γ	10^{-4}	Mannitol 2.8×10^{-1}	0.29	0.81	7.0×10^{8}	–

[HAsc] is the concentration in the ascorbic acid form and [Asc^{-}] in the ascorbate ion at pH 2. Rate constants are taken from Buxton et al. (1988).

for all proteins (higher than 10^{10} mol^{-1} L s^{-1} for a reaction of a protein with $^{\bullet}$OH) (Buxton et al., 1988). Thus, we took the values for lysozyme, another protein which also contains disulfide bonds and has no metal center. Values of the rate constant (k) of hydroxyl radical and aqueous electron for the lysozyme are 4.9×10^{10} and 4.6×10^{10} L mol^{-1} s^{-1}, respectively. Multiplying these rate constants by insulin concentration (10^{-4} M), the comparative values for $^{\bullet}$OH is 4.9×10^{6} s^{-1}. These data suggest that all the excipients studied could protect insulin against the reactive species.

Hydroxyl radicals are very strong oxidants. One of their reactions in proteins is the oxidation of tyrosine residues leading to dimers. Among protectors, the highest reaction rate constant is that of ascorbic acid. In addition, its high concentration (3.34×10^{-1} M) would enhance protection by scavenging of these free radicals, and it possesses the highest scavenging capacity (Table 2). On the other hand, mannitol and glycerol, which are poor radio-protectants against fragmentation and polymerization, also have high scavenging capacity. It seems that radio-protection is not only due to scavenging, but to more complex processes.

GSSG reacts fast with all water free radicals and was found to be a good radio-protector, especially against polymerization for which hydrogen atom and e_{aq}^{-} may play a role.

4.2. Influence of temperature

Irradiation of frozen human insulin solutions allows decreasing the insulin degradation (Fig. 7). The frozen state protects better human insulin against polymerization than against fragmentation.

According to the hypothesis of Kempner (Kempner, 2001), at very low temperatures, all the radiation damages to macromolecules are essentially due to primary ionizations occurring directly in those molecules. The dose absorbed by proteins is about 1000-fold lower than that by water molecules. Moreover, as the diffusion of reactive species of water is restricted (except that of $^{\bullet}$H), proteins are less radiosensitive and it is not surprising to find such differences regarding the yields in frozen and liquid state. The recovery of HIns after an electron-beam irradiation at 11 kGy was $84.5 \pm 6.3\%$ for frozen 10^{-4} M human insulin solution whereas after such irradiation of aqueous solutions, insulin was no longer detected.

The recoveries observed for irradiated solid solutions do not comply with the European Pharmacopoeia requirements (European Pharmacopoeia, 2005), which state that the content of insulin for injectable solution should be within 90.0 and 110.0% of the labeled insulin quantity. Cryo-irradiation (at dry-ice temperature) alone does not provide an acceptable protection.

However, a combination of these two approaches (scavengers and cryo-irradiation) produced additive effects, leading to acceptable recoveries of the therapeutic products. The best radio-protector in frozen aqueous solutions is oxidized glutathione. The recovery of HIns after an electron-beam irradiation at 11 kGy is $98.3 \pm 2.4\%$ for frozen 10^{-4} M HIns solution with 10^{-2} M oxidized glutathione. The processes that take place in radiolysis of frozen samples are poorly understood. According to literature (Karlish and Kempner, 1984; Solomonson et al., 1987; Le Maire et al., 1990; Potier et al., 1994; Filali-Mouhim et al., 1997), fragmentation does not occur at random. Oxidized glutathione protects better than reduced glutathione. Therefore, disulfide bonds present in the excipient, which reacts efficiently with reducing species, seems to increase the radio-protection.

5. Conclusion

Insulin should be administrated several times per day to diabetics. There is current research about how to improve its quality and ease of administration. Recently, a pulmonary delivery system of insulin (dry powder formulation of human insulin for pulmonary intake) has been approved (Exubera®, product of a collaboration between Pfizer Inc and Nektar Therapeutics) but injections cannot be avoided totally and the problem of sterilization at low cost should be faced. In this study, we have examined the feasibility of radiosterilization of human insulin in aqueous solution. With a dose of 10 kGy, the drug is destroyed. The two approaches used (addition of scavenger and cryo-irradiation) allowed a significant protection. The combination of these two methods allows to obtain a sufficient recovery ($\geq$90%, European Pharmacopoeia requirements) of human insulin at 10 kGy.

Acknowledgments

We would like to thank Jean Cara for having carried out the gamma irradiations, M. Descamps and Mrs. Thys (Mölnlycke Beta Plant, Waremme, Belgium) for the use of their double-beam linear electron accelerator.

References

Amareld, R.W., Wersocki, M., Drohan, M., Drohan, W.N., Burgess, W.H., Mann, D.M., Forng, R.Y., 2003. Controlled gamma-irradiation mediated pathogen inactivation of human urokinase preparations with significant recovery of enzymatic activity. Biologicals 31, 261–264.

Boess, C., Bögl, K.W., 1996. Influence of Radiation treatment on pharmaceuticals—A review: alkaloids, morphine derivatives and antibiotics. Drug Dev. Ind. Pharm. 22, 495–529.

Brange, J., Havelund, S., Hougaard, P., 1992. Chemical stability of insulin. 2. Formation of higher molecular weight transformation products during storage of pharmaceutical preparations. Pharm. Res. 9, 727–734.

Buxton, G.V., Greenstock, C.L., Helman, W.P., Ross, A.B., 1988. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($^{\bullet}$OH/$^{\bullet}$O^{-}) in aqueous solution. J. Phys. Chem. Ref. Data 17, 513–886.

European Agency for the Evaluation of Medicinal Products (EMEA), 2000. Decision trees for the selection of sterilization methods (CPMP/QWP/054/98 corr), London.

European Pharmacopoeia, 2005. Fifth ed. Council of Europe, Strasbourg.

United States Pharmacopeia National Formulary 24, 2000, United States Pharmacopeial Convention, Rockville, MD.

Ferradini, C., Pucheault, J., 1983. Biologie de l'Action des Rayonnements Ionisants. Masson, Paris.

Filali-Mouhim, A., Audette, M., St-Louis, M., Thaulette, L., Denoroy, L., Penin, F., Chen, X., Rouleau, N., Le Caer, J.-P., Rossier, J., Potier, M., Le Maire, M., 1997. Lysozyme fragmentation induced by gamma-radiolysis. Int. J. Radiat. Biol. 72, 63–70.

Gopal, N. G. S., 1995. Radiation Sterilization and Treatment of Medical Products: Current Practices, Regulations and Standards, Consultant's Meeting. Training Guidelines for Industrial Radiation Sterilization. IAEA, Jerusalem, 27–30 November.

Grieb, T., Forng, R.Y., Brown, R., Owolabi, T., Maddox, E., McBain, A., Drohan, W.N., Mann, D.M., Burgess, W.H., 2002. Effective use of gamma irradiation for pathogen inactivation of monoclonal antibody preparations. Biologicals 30, 207–216.

Karlish, S.J.D., Kempner, E.S., 1984. Minimal functional unit for transport and enzyme activities of $(Na^+ + K^+)$-ATPase as determined by radiation inactivation. Biochem. Biophys. Acta 776, 288–298.

Kempner, E.S., 2001. Effects of high-energy electrons and gamma rays directly on proteins molecules. J. Pharm. Sci. 90, 1637–1646.

Linde, S., Welinder, B.S., 1991. Non-ideal behaviour of silica-based stationary phases in trifluoroacetic acid-acetonitrile-based reversed-phase high-performance liquid chromatographic separations of insulins and proinsulins. J. Chromatogr. A 536, 43–55.

Le Maire, M., Thauvette, L., de Foresta, B., Viel, A., Beauregard, G., Potier, M., 1990. Effects of ionizing radiations on proteins. Evidence of non-random fragmentations and a caution in the use of the method for determination of molecular mass. Biochem. J. 267, 431–439.

Malencik, D.A., Sprouse, J.F., Swanson, C.A., Anderson, S.R., 1996. Dityrosine: preparation, isolation, and analysis. Anal. Biochem. 242, 202–213.

Nordhauser, F.M., Olson, W.P. (Eds.), 1998. Sterilization of Drugs and Devices: Technologies for the 2000. Interpharm Press Inc, Illinois.

Potier, M., Villemure, J.F., Thauvette, L., 1994. Radiation inactivation of proteins: temperature-dependent inter-protomeric energy transfer in ox liver catalase. Biochem. J. 298, 571–574.

Purcell, A.W., Aguilar, M.I., Hearn, M.T.W., 1995. Conformational effects in reversed-phase high-performance liquid chromatography of polypeptides. I. Resolution of insulin variants. J. Chromatogr. A 711, 61–70.

Reid, B.D., 1995. Gamma processing technology: an alternative technology for terminal sterilization of parenterals. PDA J. Pharm. Sci. Technol. 49, 83–89.

Rowe, R.C., Sheskey, P.J., Weller, P.J., 2003. Handbook of pharmaceutical Excipients, fourth ed. Pharmaceutical Press and American Pharmaceutical Association, Chicago.

Solomonson, L.P., McCreery, M.J., Kay, C.J., Barber, M.J., 1987. Radiation inactivation analysis of assimilatory NADH: nitrate reductase. Apparent functional sizes of partial activities associated with intact and proteolytically modified enzyme. J. Biol. Chem. 262, 8934–8939.

Spinks, J.W.T., Woods, R.J., 1990. An introduction to Radiation Chemistry, third ed. Wiley-Interscience Publication, John Wiley & Sons Inc, New York.

Terryn, H., Vanhelleputte, J.P., Maquille, A., Tilquin, B., 2006. Chemical analysis of solid-state irradiated human insulin. Pharm. Res. 23, 2141–2148.

Tilquin, B., 1991. Radio-sterilisation des médicaments. J. Pharm. Belg. 46, 396–398.

Tilquin, B., Rollmann, B., 1996. Recherches à conseiller pour l'application de la stérilisation ionisante des médicaments. J. Chim. Phys. 93, 224–230.

Welinder, B.S., Sørensen, H.S., 1991. Alternative mobile phases for the reverse-phase-high-performance liquid chromatography of peptides and proteins. J. Chromatogr. A 537, 181–199.

Zbikowska, H.M., Nowak, P., Wachowicz, B., 2006. Protein modification caused by a high dose of gamma irradiation in cryo-sterilized plasma: Protective effects of ascorbate. Free Radical Biol. Med. 40, 536–542.