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*Research Paper*

# Chemical Analysis of Solid-State Irradiated Human Insulin

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***Purpose.*** To study the chemical modifications induced upon irradiation of solid human insulin at radiosterilization doses and investigate the influence of the absorbed dose on radiolysis.
***Materials and Methods.*** Volatile radiolytic products were monitored by gas chromatography coupled with mass spectrometry (GC-MS) and non-volatile products by two different high performance liquid chromatography (HPLC) methods: the formation of higher molecular weight proteins was assessed by size exclusion liquid chromatography whereas assays for related compounds and chemical potency tests were carried out using reverse-phase HPLC-UV. Conformational changes were investigated by measurements of circular dichroism.
***Results.*** After gamma irradiation at 10 kGy, the recovery of insulin was 96.8%; higher molecular weight proteins accounted for 0.35% (relative peak area) and other related compounds (including $A_{21}$ desamido insulin) represented 1.29%. No major structural changes and no volatile radiolytic compounds were detected.
***Conclusion.*** Human insulin samples irradiated in the solid-state at 10 kGy (gamma rays) and 14 kGy (electron-beam) meet the European Pharmacopoeia requirements and can be considered as quite stable towards radiation from a chemical analysis viewpoint.

**KEY WORDS:** circular dichroism; HPLC; human insulin; radiosterilization; solid state.

## INTRODUCTION

Human insulin is a hypoglycemic protein hormone containing 51 amino acids divided into two chains (A with 21 amino acids and B with 30 amino acids) that are linked by two disulfide bonds ($A_7$–$B_7$ and $A_{20}$–$B_{19}$). An additional disulfide bond is also formed within chain A ($A_6$–$A_{11}$). Insulin has to be administered parenterally (intravenously or subcutaneously) as, if given by mouth it would be broken down and inactivated by the digestive system. Drugs for parenteral use have to meet the Pharmacopoeia sterility requirements. 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).

As proteins are thermosensitive, heat sterilization methods (dry heat or autoclaving) are not appropriate. Radiosterilization, as an isothermal process, could be a suitable method for the sterilization of thermosensitive drugs within the packaged product (1,2). Radiosterilization is also an effective terminal sterilization process as it allows the required Sterility Assurance Level (SAL) of $10^{-6}$ to be reached (3,4). The reference dose of radiosterilization is 25 kGy, but lower doses may be validated using appropriate sterility tests (5,6). Radiosterilization is the first choice for thermo-sensitive products and drugs in the solid state. However, radiosterilization is not considered for thermo-sensitive drugs in aqueous solutions (7).

Despite these advantages, radiosterilization does have some drawbacks. Radiolysis of solids generates radiolytic products and consequently modifies the purity profile of the irradiated drug (8). Radiolytic products are considered as impurities and can be analyzed using the International Conference on Harmonization (ICH) guidelines (9).

The present work aimed to investigate conformational changes, as well as to characterize and quantify the final products resulting from the radiolysis of solid state insulin. In order to study whether structural changes are induced upon the irradiation of solid human insulin, circular dichroism (CD) measurements in both the far and near-ultraviolet were performed. The final products were analyzed by gas (volatile) and liquid (non-volatile) chromatography. Two different high performance liquid chromatography (HPLC) methods were used: reverse-phase HPLC (RP-HLPC) for the separation of hydrophilic and hydrophobic degradation products, and size-exclusion chromatography (SEC), which allows the separation of higher molecular weight proteins.

This work is complementary to a previous article that focused on the radical mechanisms of solid-state irradiated human insulin (10).

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## MATERIALS AND METHODS

### Materials

Neat human insulin (insulin USP, human recombinant, crystalline, residual moisture 7.5%, zinc content 0.42%) was purchased from Celliance™ Corporation (a Serologicals Company) and stored in the dark at 253 K to ensure stability.

### Irradiations

The solid insulin samples were irradiated at ambient temperature in air in sealed glass vials protected from the light in a panoramic $^{60}$Co chamber (UCL—Louvain-La-Neuve—Belgium) calibrated by alanine dosimetry. The determined dose rate was 296 Gy.h$^{-1}$. The absorbed doses were 5 kGy (~17 h), 10 kGy (~34 h) and 25 kGy (~84 h).

Samples were irradiated in the same conditions with high energy electrons delivered by a double beam linear electron accelerator (LINAC) (Mölnlycke Beta Plant, Waremme, Belgium). 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 $3.2 \times 10^7$ Gy h$^{-1}$ for the single electron beam and $6.3 \times 10^7$ Gy h$^{-1}$ for the double electron beam. An internal standard, a polymethylmethacrylate (PMMA) film, was used to control the delivered dose. The measured absorbed doses were equal to 14 kGy (a single beam used, ~1.6 s) and 28 kGy (both beams used, ~1.6 s). The irradiations were repeated three times on new samples.

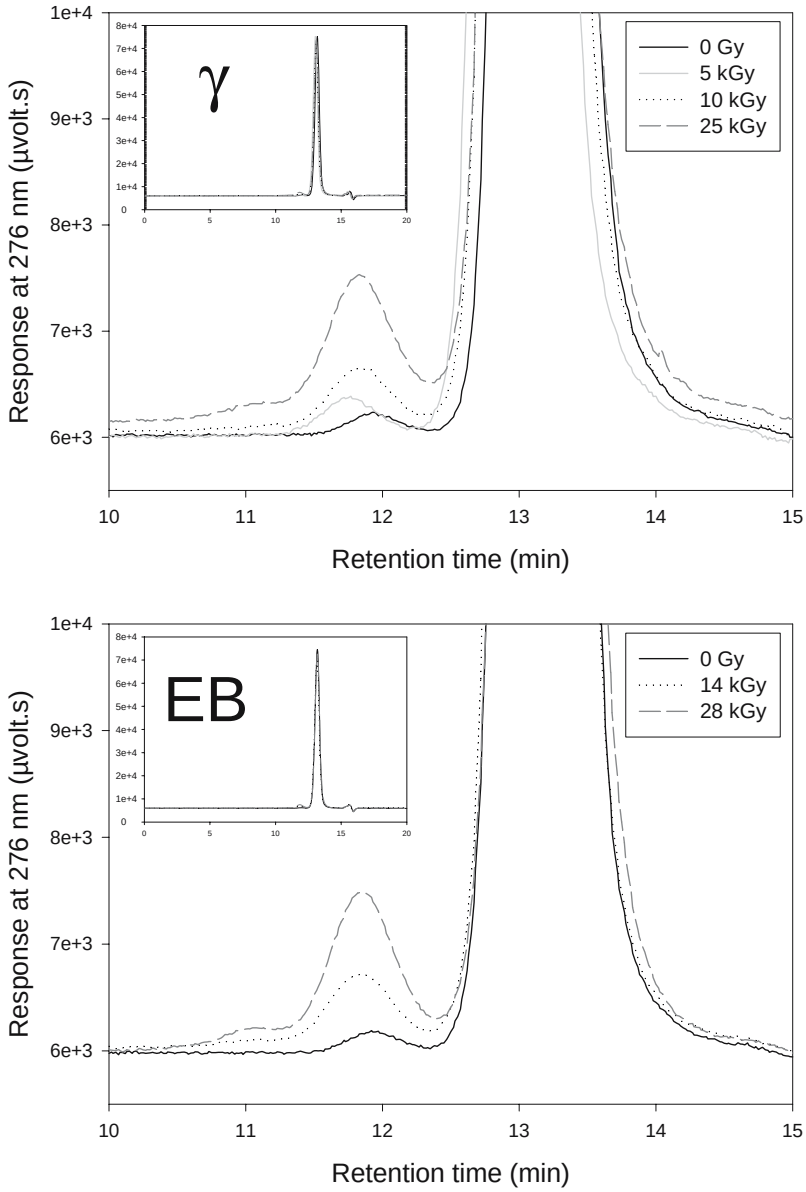


**Fig. 1.** SEC-UV chromatograms of insulin non-irradiated and irradiated at different doses. Amplification of the polymer traces. *γ* Irradiation with gamma rays. *EB* Irradiation by high energy electrons. An extended view is inserted above each zoomed chromatogram.

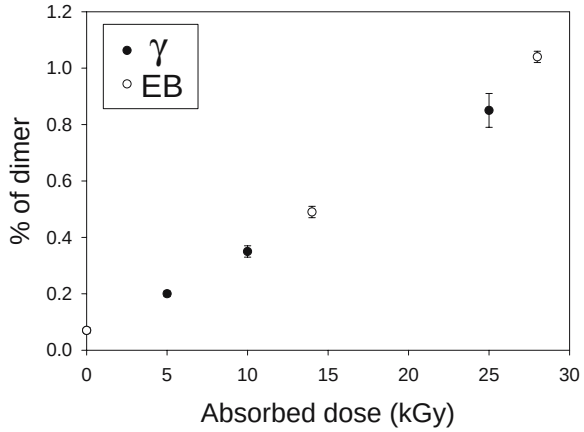


**Fig. 2.** Quantification results. Mean percentage of dimer (%) as a function of absorbed dose (kGy) from SEC-UV analysis.

## 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. The limit of detection and the limit of quantification were considered as 3 and 10 times the signal-to-noise ratio, respectively (11). Calibration curves were constructed for the quantification of the radiolytic products and for the chemical potency tests. The formation of dityrosine was monitored by measuring fluorescence at 325 nm (excitation) and 410 nm (emission) ensuring selectivity for dityrosine. Fractions that might contain tyrosine were identified by fluorescence measurements using excitation and emission wavelengths of 280 and 300 nm, respectively (12).

### *RP-HPLC Method*

A Symmetry300™ $C_4$ column, 5 µm particle size, 150 × 3.9 mm I.D. (Waters assoc., Milford, Ma) 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 (13–15). Samples were dissolved in 0.05 M phosphoric buffer (pH 2) to obtain a concentration of 2 mg/ml and were injected directly ($n$ = 3). The limit of detection was 0.01% (0.2 µg $ml^{-1}$) and the limit of quantification 0.06% (1.2 µg $ml^{-1}$).

### *SEC Method*

The SEC method proposed by Brange *et al.* (16) 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. Samples were dissolved in 0.05 M phosphoric buffer (pH 2) to obtain a concentration of 2 mg/ml and injected directly ($n$ = 3). The limit of detection was 0.01% (0.2 µg $ml^{-1}$) and limit of quantification 0.04% (0.8 µg $ml^{-1}$).

### *Liquid Chromatography Coupled with Mass Spectrometry (LC-MS) Method*

The chromatographic conditions were the same as for the RP-HPLC method. The samples were dissolved in 0.01 M HCl to obtain a concentration of 2 mg/ml. The flow was split to 0.2 ml $min^{-1}$ before entering the mass detector. A LCQ® Advantage ion trap mass spectrometer with an electrospray ionization source was used. The source voltage was 4.5 kV and the capillary temperature 200°C. The sheath gas flow was 40 A.U. Full mass scans were recorded and single ion monitoring used to track the $(M + 3H)^{+3}$ and $(M + 4H)^{+4}$ insulin ions (±1 m/z window). Instrument control and processing were performed with the Xcalibur® software.

## Circular Dichroism (CD)

Circular dichroism spectra were recorded on a Jobin-Yvon CD6 dichrograph interfaced to a computer, using a 2 mm path length for the far UV (200–250 nm) and a 10 mm path length for the near UV (250–350 nm). Data were acquired from 0.1 mg/ml sample solutions (for measurements in the far-UV region) in 0.05 M phosphoric buffer (pH 2) and from 1 mg/ml sample solutions (for measurements in the near-UV region) in 0.05 M phosphoric buffer (pH 2). Each spectrum was recorded as the result of five repeated and automatically averaged scans, using intervals of 0.5 nm and an integration time of 0.5 s at each wavelength. CD spectra measurements are expressed as mean residue ellipticity, in units of degrees square centimeter per decimole.

## Gas Chromatography Coupled with Mass Spectrometry (GC-MS)

The method developed by Barbarin *et al.* (17) was applied to non-irradiated and irradiated samples. A 0.5-gram of

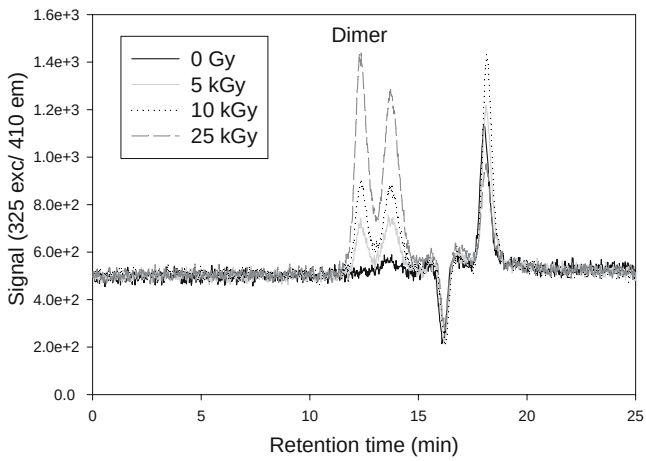


**Fig. 3.** Detection of dityrosine: overlay of the SEC fluorescence (excitation wavelength 325 nm/emission wavelength 410 nm) chromatograms of non-irradiated and gamma-irradiated human insulin.

solid human insulin was irradiated in a 10 ml vial with gamma rays (25 kGy) or high energy electrons (28 kGy) in air. Volatile compounds were analyzed by a headspace GC system with an ion trap mass detector equipped with an electron impact source. This system consisted of an HP 19395A static headspace sampler, a Finnigan TRACE GC 2000 and a Finnigan Thermo Quest TRACE MS. The GC conditions were as follows: Pora Plot Q column 25 m × 0.32 μm I.D. from Chrompack (Ea Middelburg, The Nederlands); helium at a flow rate of 1.9 ml/min was used as the carrier gas; the injection port temperature was set at 250°C; the oven temperature was maintained at 40°C for 5 min and then the temperature increased by 5°C/min to 200°C. Full mass scans from 14 to 297 m/z were performed. The ionization energy was 70 eV.

## RESULTS

### Study of the Non-Volatile Impurities

The amount of covalent aggregation, i.e., higher molecular weight proteins, was determined by SEC. This method separates proteins by their molecular shape and size. The content of insulin-related compounds was determined by RP-HPLC.

#### *Aggregate Formation (SEC)*

An overlay of SEC-UV chromatograms of human insulin, non-irradiated and irradiated at different doses with gamma rays and high energy electrons is shown in Fig. 1 The

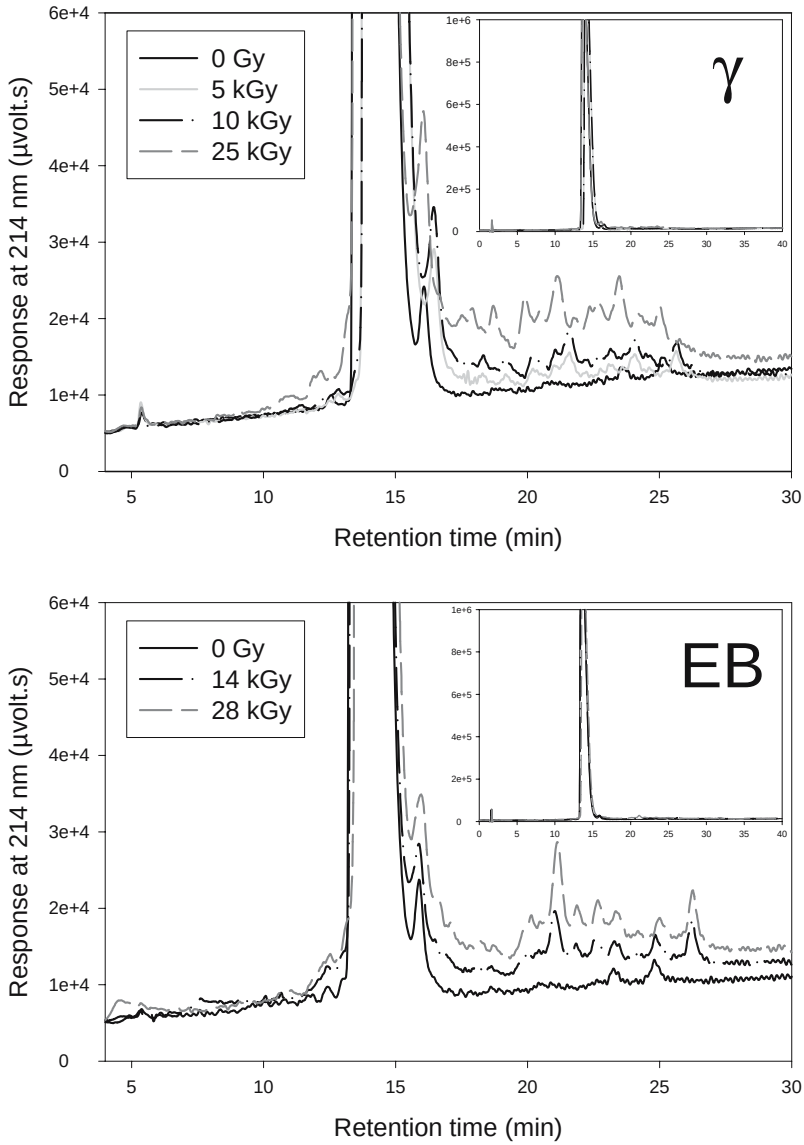


**Fig. 4.** Overlay of the RP-HPLC-UV chromatograms zoomed on the degradation product peaks for solid insulin non-irradiated and irradiated at different doses. *γ* Irradiation with gamma rays. *EB* Irradiation by high energy electrons. An extended view is inserted above each zoomed chromatogram.

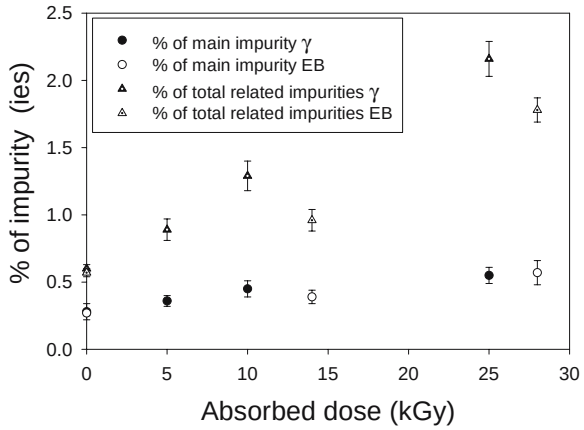


**Fig. 5.** Quantification results. Mean percentages of main impurity and total related impurities (%) *versus* absorbed dose (kGy) for RP-HPLC-UV analysis.

peak corresponding to the dimer is quantified (Fig. 2) after gamma ray and electron beam irradiations at different doses. A small percentage of dimer (0.07%) was present prior to irradiation. According to the certificate of analysis, the percentages of dimer and polymer impurities present in the drug substance have to be less than or equal to 0.3 and 0.05%, respectively. For both gamma ray and electron beam irradiations, the dimer concentration increased with the irradiation dose. After irradiation, the fluorimetric detector (325 nm excitation/410 nm emission) showed a peak at the dimer retention time (Fig. 3), which increased with the absorbed dose. At the highest doses (25 and 28 kGy), a new peak is detected at 11 min, corresponding to insulin polymers. This peak is below the quantification limit and, therefore, cannot be quantified.

*Related Compounds (RP-HPLC)*

The chromatograms (RP-HPLC-UV) of the human insulin before and after irradiation with gamma rays and high energy electrons are presented in Fig. 4. After irradiation, differences in the chromatographic profile were observed. Some degradation products that were already present before irradiation, increased with the absorbed doses (e.g., the main impurity with retention time 16 min) and numerous new radiolytic products were detected in very small amounts (traces). The diode array spectra of these products were similar to that of human insulin. The fluorescence detector allows the peak at retention time 5.5 min to be identified as a dityrosine. The dityrosine peak was already present in the chromatogram of non-irradiated insulin and increased after gamma ray but not after electron-beam irradiations. At this retention time, no fluorimetric signal was recorded ($\lambda$exc 280 nm/$\lambda$em 300 nm), which confirms the absence of tyrosine in this fraction.

The purity of the peak of human insulin was confirmed by LC-MS which showed that the mass spectra recorded at the beginning and at the end of the peak were similar. The $(M+3H)^{+3}$ ($m/z$ 1,936) and $(M+4H)^{+4}$ ($m/z$ 1,453) are two major ions (18). The diode array detector indicated a peak purity of 1.000 for the peak of human insulin in the chromatogram of both electron beam and gamma-irradiated samples. This shows that no product coelutes with human insulin peak.

The recovery of human insulin after a gamma irradiation at 10 kGy was 96.8 ± 1.5%, and after an electron-beam irradiation at 14 kGy was 96.4 ± 0.6%. These values comply with the requirements from the European Pharmacopoeia (19) which state that the content of both human insulin and $A_{21}$ desamido-human insulin should be within 95.0 and 105.0%.

The percentages of the impurities present before irradiation, and those of the radiolytic compounds were determined. As recommended by the ICH guidelines (9), the molar absorptivities of the degradation products are assumed to be identical to that of human insulin and thus, the response factor of human insulin was used. The concentration of each impurity is expressed as a percentage of the main compound (human insulin). Figure 5 shows the percentage of total impurities and the percentage of the main impurity (retention time close to 16 min) as a function of the absorbed dose for gamma and electron beam irradiations. Some impurities were detected before irradiation. Based on the analysis certificate of human insulin, the percentage of total related compounds must not exceed 2%. After a 10 kGy gamma irradiation, 0.35 ± 0.02% (relative peak area) of dimers and 1.29 ± 0.11% of other related compounds (including $A_{21}$ desamido insulin) were detected.

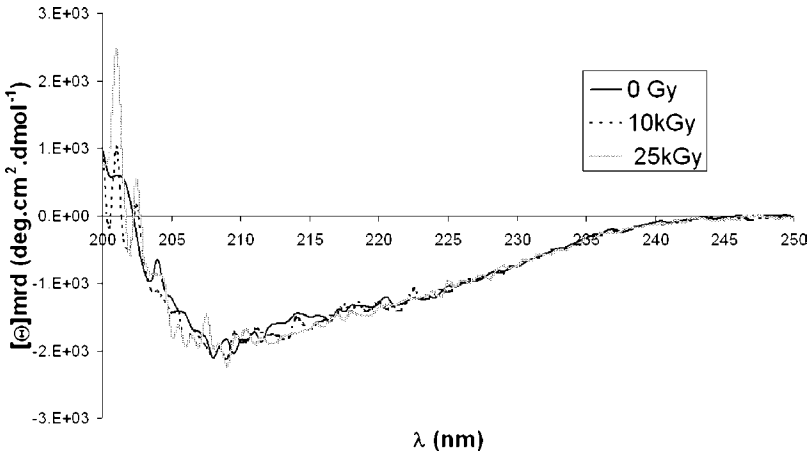


**Fig. 6.** Far-UV CD spectra of human insulin irradiated with gamma rays at various doses.

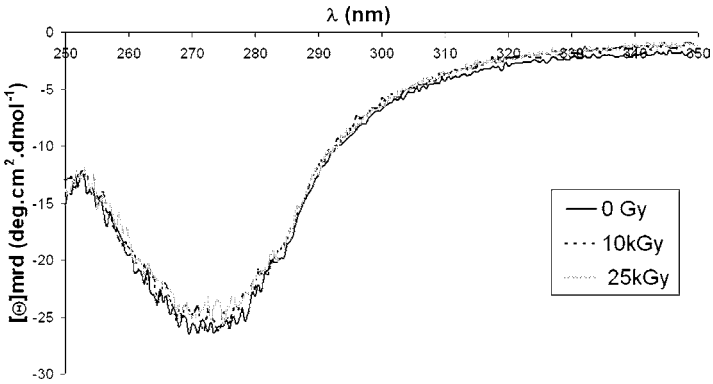


**Fig. 7.** Near-UV CD spectra of human insulin irradiated with gamma rays at various doses.

### Study of Conformational Changes (CD)

*Far-UV*

Far-UV CD spectra of gamma-irradiated human insulin were recorded as a function of the irradiation dose (Fig. 6). In the far-UV region, the spectrum is dominated by the electronic transition of the peptide bond, in particular α-helix produced the most intense signal with two negative maxima near 208 and 222 nm (20–22). No significant differences were observed between the CD spectra before and after irradiations with gamma rays or high energy electrons, which shows that the absorbed dose and the irradiation type do not influence the CD spectrum.

*Near-UV*

Figure 7 shows the near-ultraviolet CD spectra of human insulin before and after gamma irradiation at various doses. The peak at 276 nm seems to decrease slightly on irradiation. No significant differences were observed between electron beam and gamma irradiations.

### Study of the Volatile Impurities

Volatile compounds generated by radiolysis may be identified by gas-chromatography with mass spectrometry detection (GC-MS). A static headspace sampling method was used (17,23). No difference was observed in the chromatograms before and after irradiation: no new peak was detected and there was no modification of the pre-existing peaks (Fig. 8). Irradiation did not produce any detectable volatile products.

## DISCUSSION

### Study of the Non-Volatile Impurities

RP-HPLC and SEC have proved to be valuable tools for the separation of insulin and its radiolytic products. Irradiation with gamma rays and high energy electrons induces dimerization, and polymers are even observed at the highest absorbed doses. Dimerization increases with the absorbed dose. There was no influence of dose rate on dimer formation (within the studied dose-rate range).

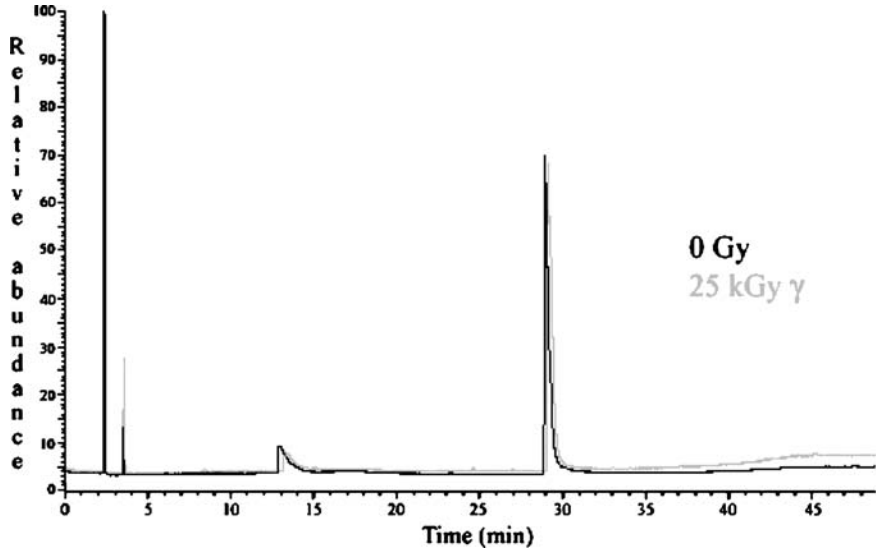


**Fig. 8.** Overlay of non-irradiated and γ-irradiated (25 kGy) GC-MS chromatograms for solid human insulin.

The literature on the chemical stability of insulin during storage (24) reports that covalent insulin dimers are formed through a transamidation reaction, mainly between the B-chain N-terminal and one of the amide side-chains of the A chain ($Gln^{A15}$,$Asn^{A18}$, $Asn^{A21}$). In this case, the increase in the fluorimetric signal at the dimer retention time after irradiation seems to indicate that dityrosine may play a role in insulin aggregation. The Electron Paramagnetic Resonance (EPR) study (10) has shown that the irradiation of solid state insulin generates perthiyl (RSS°) so that some dimers induced by irradiation may be due to disulfide interactions (disulfide lysis).

The detection and the separation of related radiolytic products is a challenge since they are numerous, similar and present in traces. Radiolytic products can either arise from the production of a new compound or from an increase in a pre-existing impurity (e.g., the main radiolytic product). The elution order of close related peptides may be explained by the difference in the hydrophobicity of the amino acid residues (25). The majority of radiolytic products are more hydrophobic than the insulin molecule as they show more affinity for the stationary phase. The differences may be attributed either to conformational changes due to the melting of the insulin molecule tertiary structure or to the chemical degradation of the solute (25). For example, deamidation removes $NH_3$ and, therefore, the residue is COOH which causes the corresponding peptide to be less polar as, at low pH, R-$NH_3^+$ is more polar than R-COOH.

The total related compounds and the main impurity increased with the dose for both electron beam and gamma ray irradiations.

After gamma irradiation at 10 kGy, all the radiolytic products were detected in concentrations below 0.1% except one that was above this threshold value. Following the ICH guidelines (9), the identification of this product would be required if insulin had to be radiosterilized. This main impurity was already present before irradiation and, according to the analysis certificate and insulin stability data (26–29), it corresponds to $A_{21}$ desamido-insulin (deamidation on the asparagine 21 residue of chain A). Regarding its activity, deamidated insulin shows almost the same potency as insulin itself (27,28).

### Study of Conformational Changes

Circular dichroism provides distinct information on both tertiary and secondary structures and is a useful tool for monitoring changes in protein conformation.

In the far-UV, CD measurements detect transitions primarily involving the peptide chromophores and are sensitive to secondary conformation (30). The results obtained from far-UV CD measurements show that the irradiation of human insulin does not modify the intensity of the bands at 208 and 222 nm, and, therefore the α-helix content in the hormone secondary structure is not modified.

Circular dichroism bands of proteins in the near ultraviolet are associated with optically active side-chain chromophores, mainly aromatic residues and cystine. These chromophores absorb around 270 nm (31). Insulin has no tryptophane but contains four tyrosine, three phenylalanine residues and three disulfide bonds that together contribute to the absorption spectrum. Previous studies have investigated the contribution of the tyrosine residues to the CD spectrum of insulin (32–35). The slight intensity decrease at 276 nm could arise from a modification of the tertiary structure or from a radiolytic breakage of the chromophore (tyrosine). This last proposal is in agreement with the fluorescence results. The CD results show that a solid state irradiation at radiosterilization doses perturbs the secondary and tertiary structures of human insulin very little or not at all. However, some effects have been reported for other proteins such as α-lactalbumin (36).

### Study of Volatile Impurities

Headspace GC-MS has previously allowed the detection of volatile radiolytic products from the irradiation of solid antibiotics. According to Barbarin *et al.* (17), various routes might explain the formation of volatile radiolytic products: they could arise from the molecular degradation of drugs, from the residual solvents that are present in the non-irradiated drugs, or from the reaction of radicals between drug and solvents. However, in the case of solid human insulin, irradiation does not produce any detectable volatile products (small fragments). The absence of volatile radiolytic products might be explained by the internal distribution of energy at high speed in a large molecule. The literature (37) suggests that the absorbed energy is dispersed through the solid matrix which explains why minor chemical changes are induced in molecular solids. In accordance with previous work (17), the amount of volatile products in irradiated solid insulin should be less than 1 ppm so that they are not detected.

## CONCLUSION

A previous study (10), on the radiolytic yields of 10 kGy gamma-irradiated insulin, has shown the radioresistance of solid insulin. The various analytical techniques used in the present study confirm the stability of solid human insulin towards electron beam and gamma irradiations. After irradiation, the content of human insulin meets the European Pharmacopoeia requirements and small amounts of various degradation products are detected. Therefore, ionizing radiation may be considered as a potential method for the sterilization of human insulin in the solid state (especially at low doses, e.g., 10 kGy).

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