

Radiosensitivity Study of Freeze-dried Antibodies to Gamma Irradiation

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Abstract

Analysis of degradation freeze-dried antibodies by ⁶⁰Co gamma irradiation was performed using the UV Scanning Spectroscopy, Gas Chromatography, High Performance Liquid Chromatography and Electron Paramagnetic Resonance techniques. The byproducts formed after irradiation are in very low abundance. They were not detected even when using high sensitivity GC and UV Scanning Spectroscopy. We also anticipate that the formed byproducts will be similar to the non-damaged product. The EPR spectra of all monoclonal antibodies are asymmetric features centered around 332 T. The signal intensity depends on the irradiation dose linearly up to 15 kGy, while at higher doses the linearity disappears apparently by radical recombination despite the low diffusion properties of the freeze-dried compounds. The most radiosensitive antibodies were humanized monoclonal antibodies and the glucose played a radioprotector role. The HPLC results indicate that it is an adequate technique to detect the byproducts. The formed radical are very stable and they can be detected up to nine months.

Key words: gamma radiation, antibodies, radical stability

Introduction

The regulations for radiosterilization are different among countries, but with the publication of ANSI/AAMI/ISO 11137 by American National Standard at least there now exists a recognized guidance for implementing this technology. The publication describes specific requirements for validation, process control and routine monitoring in the radiation sterilization of health care products. This guidance may be applied to continuous and batch type gamma irradiators, using the radionuclides ⁶⁰Co and ¹³⁷Cs, and also to irradiators using a beam from an electron or x-ray generator.

Gamma radiation is generated by the spontaneous decay of radioisotopes. Exposure to gamma rays sterilizes the product by disrupting the DNA structure of microorganisms located on or within the product, thereby eliminating its ability to reproduce life-sustaining cells. The deep penetration of gamma rays makes it an ideal solution for products having various densities and types of product packaging. The gamma ray sterilization process is reproducible, easy to use, validate and has a proven track record. Using dosimetric release procedures allows for the shipment of products immediately after the sterilization processing [1].

On the other hand, during the irradiation of the biopharmaceutical products there occurs the appearance of free radicals, with a high chemical reactivity that can induce changes in the pharmacological, organoleptical and toxicological drug properties [2-4]. These new byproducts are usually at a very low concentration in drugs, approximately 10⁻⁷ M [5].

A suitable technique for the detection of free radicals should be specific for the radiation treatment. The sensitivity of the technique should be high enough to allow signal recording over the self life of the product. The most common techniques we found useful for providing qualitative and quantitative data, whether or not sample has been irradiated, were UV-Spectroscopy, Gas Chromatography High Performance Liquid Chromatography and Electron Paramagnetic Resonance techniques [6].

The objectives of this study are to establish the techniques to discriminate between irradiated and nonirradiated freeze-dried antibodies and to evaluate the stability of the formed radiolitical byproducts.

Materials and Methods

Drugs

For our study, we have used formulations containing two murine monoclonal antibodies obtained from mouse liquid ascitic (ior/egf r3 and ior ceal), one chimeric (t3Q) and one humanized monoclonal antibodies (hr3) produced from bioreactor supernatants in the Center of Molecular Immunology in Cuba. The antibodies were freeze-dried and sealed under vacuum.

γ-Irradiation

The dried products were irradiated with gamma rays source of ⁶⁰Cobalt at room temperature. The dose rate was 2.17 kGy, while the selected doses in Cuba were: 5, 10, 15, 20 and 25 kGy. A second part was irradiated in Belgium at 3 y 13 kGy at ambient temperatures and under liquid nitrogen in Belgium. Doses were first confirmed by chemical or physical dosimeters in irradiation equipment. A nonirradiated sample was kept as a reference.

UV Scanning Spectroscopy

After irradiation, the UV adsorption spectrum in the range of 200 to 400 nm was measured at one and six month intervals. The measure-

LYOPHILIZATION

ments of absorbance at one month were performed in Belgium at $\lambda=200 - 400$ nm using a double-beam spectrophotometer and after six months in Cuba using a UV/Visible spectrophotometer, having a range of 190 to 1100nm through Determination Coefficient R^2 .

Gas Chromatography (GC)

The system to identify the volatile compounds by Chromatography in the gaseous phase includes the following equipment:

- Accelerated Purge and Trap Sample Concentrator
- Liquid autosampler (use a capillary column DB-XLB, 0,25 mm, 25 m)
- The mass detector with a source EI (electronic impact)

The whole GC-MS equipment is commanded by the logician data system software. The carrier gas used was helium.

The Electronparamagnetic Resonance

Ten EPR spectra for each sample were recorded from room temperature and up to 373 K using the cw X-band spectrometer. Samples were filled in standard quartz tubes. The DPPH (2,2 diphenil-1-picrylhydrazyl) quartz tube was used as an EPR quantitative reference, $g=2.0036$. The EPR signal/dose curves were followed by recording the signal amplitude height of the control line of the spectrum and the signal area (DI/N) determined by the double integration of the derivate spectral curves. The center field was established at 332 T and the size field 30 T. The microwave frequency was 9.33 GHz and at 100 Hz modulation frequency.

The g-factor was determined by the formula: $g = hv/\beta eB = v(\text{MHz})/13.99614 \text{ B (T)}$

1 Tesla (T) = 10 Gauss (G)

g Constant that depend of radical electronic configuration

v frequency

B magnetic field

β Böhr's Constant

High Performance Liquid Chromatography (HPLC)

SEC-HPLC was performed at 1 mL/min using 0.1M phosphate buffer, pH 7.2.

100-500ug (100uL) by injected into a TSK G3000SWxl column using an autosampler. Absorbance was measured at 280 nm wavelength. Analysis software and HPLC system were used. The column was previously calibrated, and monomer, dimmers and polymers in the sample were quantified. High Performance Liquid Chromatography was carried out in the HPLC equipment with a TSKG 3000 column at 280nm.

Results and Discussion

UV Scanning Spectroscopy

We have not observed modifications in the color and smell of the biopharmaceutical products after irradiation as some authors have reported [2-4], the only modification (significant change) that we observed was in the color of the vial glass (typical darkening). This change in color of the glass should not be confused with differences between the irradiated and nonirradiated product and for this reason we did not considered the color change to be a key factor.

Table I. Values of determination coefficient R^2 obtained by UV Scanning Spectroscopy relative to the control

Doses (kGy)	1 month	6 months
5	0.9997	0.9998
10	0.9990	0.9995
15	0.9957	0.9973
20	0.9944	0.9962
25	0.9892	0.9962

From the results of Table I, it is clear that there is no statistically significant difference in the irradiated humanized monoclonal antibody-the most radiosensitive of the studied antibodies- and the nonirradiated controls during the time of study (up to six months). Similar results were obtained for other antibodies tested.

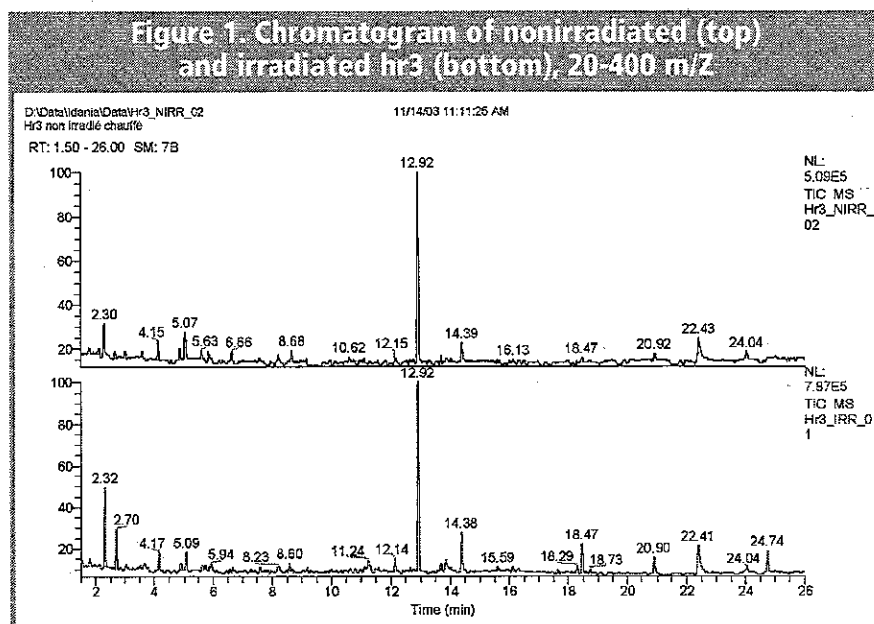
These results confirm that the changes in vial color were not associated with a change in the freeze-dried product. These results suggest that the changes previously reported in the color and smell of some pharmaceutical products were to residual solvents used in formulating the radiolysis product [2-4,6]. However in our case, we did not use these organic solvents in the manufacturing process. Because of the high sensitivity of the analytical techniques used, these results also confirm that the byproducts formed after irradiation are in very low abundance. We also should expect, but have not confirmed, that the formed byproducts are very similar to the non-damaged product both from the point of view of their structure and pharmacological characteristics.

Gas Chromatography (GC)

From Figure 1, we observe there are not significant differences between the irradiated and nonirradiated products, and thus this explains the absence of any odor in our irradiated products. This fact confirms the absence of residual solvents in the manufacturing process.

The Electronparamagnetic Resonance

For solid phase compounds, the EPR spectra are always poorly resolved. This results from the overlapping of the different spectra



that are related to the different directions of initial spins with regard to the magnetic field [8-11].

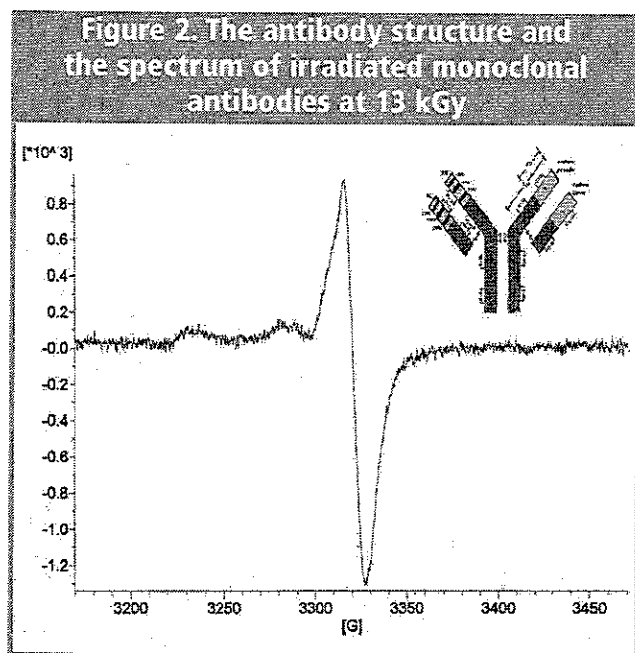


Figure 2 presents the typical asymmetrical EPR spectrum obtained for antibodies after gamma irradiation. The spectrum is composed of a broad doublet of separation approximately 5 T (g-factor between 2.03 and 2.06) approximately at 323 and 328 T and one more or less symmetric signal centered at 332 T with g-factors between 2.003 and 2.005. We did not detect any signal in the EPR analysis of the non-irradiated monoclonal antibodies. All this suggests the presence of different kinds of radicals (that were confirmed by the stability study at different powers, the results of which are not shown) and further supports that the EPR studies have not thrown much light thus far on the mechanism of the decomposition of biological systems. Because the hyperfine spectra are generally poorly resolved, it appears from the g-factors that the peaks at 323 and 328 T are related to sulfur-containing radicals (the antibodies are rich sulfur-containing compounds, see Figure 2), while the higher peaks more probably are related to carboxyl or hydroxyl radicals [9-10].

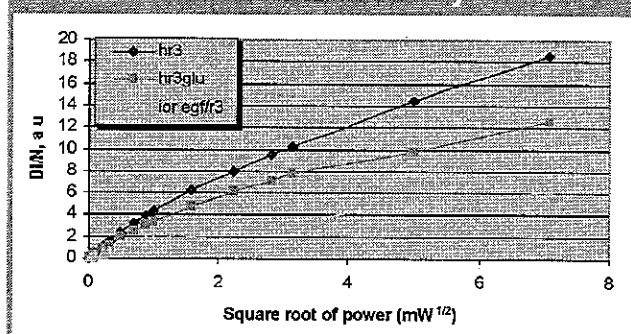
The curved shapes obtained for humanized, chimeric and murine antibodies do not have different sensitivities to radiation, but there are great differences in the pick amplitudes. This fact implies that each antibody has a different sensitivity to radiation.

Figure 3 shows the differences in the adsorption surface of humanized and murine irradiated monoclonal antibodies. From this study, we surmised that the humanized monoclonal antibodies were the most radiosensitive chimeric and murine monoclonal antibodies. We therefore can also conclude that the glucose supplement exerts a radio-protective effect over the humanized monoclonal antibody. For all studied antibodies, we obtained an evolution of the dose-EPR response curve after radiosterilization that increased linearly until 15 kGy as square root of the microwave power.

From this dose level appear two inflexions in the slope of the big peaks attributed to electronic delocalizations, it seems that at high doses begin to occur as a result diffusion or recombination of radicals in the solid matrix structure [2,10]. It is confirmed that once the quartz tube is exposed to radiation, the slopes disappeared, but the g-factor remained the same values.

The recorded EPR spectra for our product did not show any differences in the radicals formed, despite the effects of heat, UV or gamma irradiation. However, the fact that the formed radicals were of

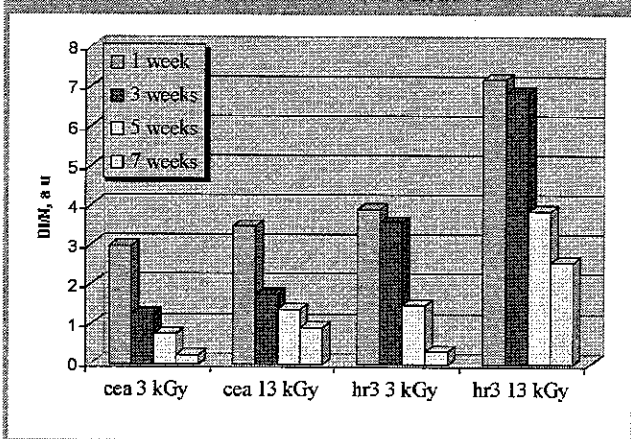
Figure 3. Adsorption surface of irradiated antibodies at 25 kGy



the same type does not necessarily imply that the mechanisms involved in their formation were the same in all cases.

The values of g-factor obtained for very stable radicals are not affected by UV, high temperature (until 100°C) or the time. Nevertheless from Figure 4, we can appreciate that if the nature of radicals remains the same, the quantity decreases with the time, but yet they are detectable nine months after irradiation. We can conclude that γ -irradiation produced some very stable free radicals in our freeze-dried antibodies. We did not observe significant differences between the irradiated products under liquid nitrogen and at room temperature. In principle, it is attributed to a freeze-dried product that has a frozen state, where the mobility of the water in the glassy interstitial region approaches 0 and the formulation is considered completely frozen [12].

Figure 4. Decay of adsorption surface of irradiated antibodies FF



High Performance Liquid Chromatography (HPLC)

Table II shows the results obtained by molecular size exclusion analysis using HPLC for the humanized antibody with glucose presence in the formulation.

This method resulted to have higher sensitivity to detect the effect of radiations in the antibodies.

In this study, we detected that already at a dose level of 5 kGy there was the induction of antibody fragmentation over the 5%. These values are higher than those established for the products in agreement with the quality specifications.

Once more we can expect that the formed byproducts are very similar to the non-damaged product from the point of view of their struc-

Table II. Purity of humanized freeze-dried antibodies after irradiation

Doses (kGy)	Retention peak (%)	
	5.6 min	6.2 min
0	94.00	4.30
5	88.79	5.61
10	83.64	6.66
15	76.20	7.55
20	71.59	7.71
25	65.49	7.88

ture and pharmacological characteristics. It is necessary to point out that the EPR analysis is able to detect trapped radicals, which are the precursors of the radiolytical products that are detected by the HPLC and UV Scanning Spectroscopy. Once the product has been dissolved in water, it is probable that we can obtain different radical yields at different residual moisture levels or freeze-dried cake quality. The trapped radicals in the irradiated freeze-dried products are very stable radicals, but their concentration does decrease with time. Nonetheless, their presence is still detected after nine months which confirms the previously reported long radical life [13-14].

Conclusions

The studied monoclonal antibodies did not show organoleptical changes in the color or smell resulting from residual organic solvents. The techniques of UV-Spectroscopy and Gas Chromatography did not detect differences between the irradiated products and nonirradiated products. However, the High Performance Liquid Chromatography and Electron Paramagnetic Resonance techniques did give us qualitative and quantitative data of formed radicals that suggest the possibility to use them in postdosimetry analysis as well. The humanized monoclonal antibodies were the more radiosensitive to gamma radiation than chimeric and murine antibodies, while the glucose presence in the formulation plays a radioprotector role. The trapped radicals in the irradiated freeze-dried products are very stable radicals, but their concentration does decrease with time. In spite of this, their presence is still detected after nine months.

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