Investigating Potential Effects of RFID Systems on the Molecular Structure of the Human Insulin

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Abstract—The Radio Frequency Identification (RFID) is a wireless technology that is becoming more and more important as auto-identification solution for many application scenarios. The adoption of this innovative technology in the pharmaceutical sector promises to solve several problems related to tracing and tracking systems at item level. Unfortunately, there are still some barriers limiting the large-scale deployment of RFID technologies. One of these is related to very interesting research topics on the evaluation of potential effects of electromagnetic fields on drugs. In detail, this work aimed to analyze the impact of UHF RFID devices, used in tracing systems, on the molecular structure and potency of a commercial human insulin preparation, ActrapidTM. In order to investigate possible induced alterations of molecular structure, the Reverse Phase-High Pressure Liquid Chromatography and the Nuclear Magnetic Resonance spectroscopy have been mainly used in the experimental protocol. To obtain some indications about drug performance, *in vitro* cell proliferation assays have been also conducted. The experimental results, achieved by a protocol combining an accurate structural analysis on 5 min to 24 h irradiated drug samples with functional *in vitro* assays, have shown that the electromagnetic field generated by UHF RFID devices does not cause significant effects on ActrapidTM insulin. These findings are strongly encouraging the use of RFID-based technologies for item-level tracing systems in the pharmaceutical supply chain.

I. INTRODUCTION

THE item-level tagging is one of the main challenges of many application scenarios, such as manufacturing, logistics, ticketing, and supply chain management. The pharmaceutical supply chain, with millions of medicines moving around the world each year that have to be traced at item-level, represents a very interesting application scenario. Its fragmentation, caused by the overwhelming growth of intermediate wholesalers and retailers involved in drug flow, results in a decreased supply chain transparency and increased difficulties to track and trace medicines. Furthermore, the growing counterfeiting problem [1] raises a significant threat within the supply chain system.

Currently, the auto-identification solutions mainly used in the pharmaceutical sector are based on optical technology such as linear or bi-dimensional (i.e. DataMatrix) bar codes. Unfortunately, these solutions are not effective for tracing systems on the supply chain because they require a line-of-sight communication, cannot be written or read in bulk, can be easily counterfeited, can limit the speed of packaging line operations, etc. The RFID technology [2] is a very attractive auto-identification solution and the great strides made in this field are going to overcome or limit a lot of the cited problems. In particular, the choice based on passive Ultra High Frequency (UHF) RFID systems is able to guarantee advantageous aspects such as the capability to enable multiple simultaneous readings of tags also in non line-of-sight conditions, offer very high read rates, and allow a long distance between reader and tag (i.e. transponder). These characteristics lead to consider UHF band as the ideal choice for item-level tracing applications. Some FDA recommendations [3] invited the main actors of the pharmaceutical supply chain to experiment, through pilot projects, the use of RFID technologies in order to develop efficient item-level tracing systems.

Unfortunately, there are still some barriers that are limiting the deployment of these technologies in the pharmaceutical sector. Among these, the research community is focusing on technology weaknesses (e.g. high read rate and data reliability in critical conditions) [4, 5], security issues (i.e. data access, privacy and legislation), and absence of an exhaustive evaluation of the effects of the RFID systems on drugs.

Some previous works [6] have just started to evaluate the impact of RF exposure on biological drugs. They focused on possible alterations of the molecular structure of ActrapidTM insulin by using, as investigative techniques, HPLC chromatography and NMR spectroscopy, but these studies did not clearly demonstrated the absence of effects on drug structure and performance because of the presence of excipients and low drug concentration.

In this paper, an improved experimental protocol has been adopted in order to complete the analysis on ActrapidTM insulin preparation. To improve the accuracy of protocol and limit problems related to the variability of insulin preparations from different production lots, 8 samples (4 control and 4 exposed to RF radiation) were investigated for each of three different lots, for a total of 24 samples. Moreover, different experimental conditions (e.g. deuterated solvent, concentration) have been applied for the NMR samples with respect to those previously reported [6]. Cellular proliferation assays have been performed to better define the potential variations of drug function. Conclusively, further NMR spectra were recorded from pure insulin samples. All data obtained by merging of the

analytical techniques (HPLC and NMR spectroscopy) and cell proliferation tests, suggest that the effects of RF emissions on drug quality were negligible and strongly encourage the use of RFID-based technologies for item-level tracing systems in the pharmaceutical supply chain.

The rest of the paper is organized as follows. The Section II reports materials and methods used in the experimental protocol. The main results obtained by a combined analysis based on the use of three different investigative techniques are discussed in Section III. Finally, Section IV reports conclusions and suggests some open issues.

II. MATERIALS AND METHODS

A. RFID Test Environment

In order to conduct effective experimental campaigns to evaluate the potential effects of UHF RFID technologies on biological drugs, a particular test environment, reproducing the main steps (e.g. items line, cases line, and border gate) of the pharmaceutical supply chain, has been realized in laboratory. In the experimental protocol, the cases line was chosen as exposure place because it is characterized by considerably stressful operating conditions. In fact, the values of electric and magnetic fields are higher than those measured in the other steps. The "cases line" consists of a conveyor belt, equipped with a UHF RFID reader, the Impinj's Speedway. Its operating frequency range is 865-956 MHz. Its maximum transmission RF power is +30 dBm (1 Watt), respecting the rules of most countries. Furthermore, the line is characterized by a metallic tunnel equipped with four near field UHF antennas of the same type: the Impinj's CS-777 Brickyard. Each reader antenna is in the centre of each tunnel side. The width of the tunnel is 0.6 m. The reader antennas are suitable for 865-868 MHz frequency range. The antenna diameter is 0.30 m.

In order to obtain some indications about the intensity of the electric field inside the tunnel, where the drug sample is located to be irradiated, the PMM 8053A analyzer and the probe PMM EP-330 were used. The average and peak values of the electric (E) field measured during the exposure period are respectively 30 and 41 V/m Furthermore, let us observe that during the exposure phase, the temperature was uniform (25°C). This test environment has been used to carry out the first part of the experimental protocol. The distance between drug sample and reader antenna is about 0.3 m. In the exposure phase, no RFID tag is applied on the drug sample. This assumption is valid because the effects of the electromagnetic field generated by one RFID tag for backscattering is negligible respect to electromagnetic field generated by reader antennas.

B. Exposure Experimental Campaigns

Twelve vials from three different lots (four from every batch) of ActrapidTM human insulin (100 I.U. ml^{-1}) were analyzed. The content of each vial (10 mL) was separated into two 5 mL aliquots. Only a fraction was exposed to the electromagnetic field generated by RFID devices in UHF band, 1 W transmission power. In this way, both the untreated and RF exposed samples were taken from the same vial. The experimental protocol used four different exposure periods (5 min, 1 h, 6 h and 24 h) for each lot. In conclusion, the total number of Actrapid samples was 24 (i.e. 12 control and 12 irradiated samples). Both types of samples (control and irradiated) were analyzed by using the preselected investigative techniques (i.e. RP-HPLC, MTT assay, 1D and 2D NMR spectroscopy).

C. Biological Materials

Chemicals and Reagents. ActrapidTM (100 I.U. ml^{-1}; Lot numbers XS63630, XS63631, XS64223) was purchased from Novo Nordisk (Bagsvaerd, Denmark). Diethyl ether, bidistillated water, deuterated acetic acid (CD_3COOD) and recombinant human insulin were purchased from Aldrich and used without further purification. CH_3CN (HPLC grade) and extra-pure K_2HPO_4 were from Merck (Darmstadt, Germany). Cell culture media, fetal bovine serum (FBS), L-glutamine, antibiotics and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Euroclone Life Science (Pero, MI, Italy). Hormones were obtained from Sigma Aldrich (Milan, Italy).

Cell Culture. Rat PCCl3 thyroid cells were cultured in Coon's modified Ham's F12 medium, supplemented with 5% FBS, a mix of six hormones [1 µg/ml insulin, 3.62 µg/ml hydrocortisone, 5 µg/ml apo-transferrin, 20 ng/ml GHL, 10 ng/ml somatostatin, 1 mU/ml TSH (thyroid stimulating hormone)], 2 mM L-glutamine, 0.05 mg/ml gentamicin, 5000 U/ml/5 mg/ml penicillin/streptomycin. Human MCF7 breast carcinoma cells were grown in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% FBS, 2 mM L-glutamine and 5000 U/ml/5 mg/ml penicillin/streptomycin.

D. Investigation Methods

Nuclear Magnetic Resonance (NMR). High resolution NMR spectroscopy is suitable to assess molecular structures, time dependent phenomena, such as kinetic reaction and inter- and intramolecular dynamics, and detailed structural analyses of many molecules among which peptides and proteins. Specially, due to the developments in the spectrometer technology (high field instruments and cryoprobes) the NMR spectroscopy is the useful method to study molecular recognition and protein folding in solution analysing typically the ^{1}H, ^{13}C, ^{15}N nuclei [6, 7].

The NMR samples of pure insulin were prepared dissolving the protein directly in a mixture of water and deuterated acetic acid (80:20 vol %) in order to obtain a 2.5 mM concentration. For the NMR analysis of commercial insulin preparation, 2 mL from every 24 samples of Actrapid were treated with diethyl ether (20 mL) in order to remove the majority of excipients (*m*-cresol and glycerol) from the pharmaceutical preparation. The aqueous residue was then lyophilized and dissolved in 0.4 mL of H_2O/CD_3COOD

80/20 vol % mixture and placed in a 5 mm NMR tube. 1D ^{1}H NMR, 2D ^{1}H COSY, ^{1}H NOESY and ^{1}H TOCSY water suppression spectra were recorded at 298.15 K on a BrukerAvance III 400MHz. Chemical shift values were referenced to DSS (5,5-dimethylsilapentanesulfonate) indirectly. DSS was added to the sample only after the 2D spectra were collected. The spectra were acquired and processed using the software Topspin 2.1 (Bruker Biospin). In all 1D and 2D spectra the water signal was suppressed by a watergate or presaturation sequences. 1D ^{1}H NMR spectra were acquired with 32K data points, spectral width of 4795.396 Hz, 1000 scans with a 2 s repetition delay. 2D ^{1}H COSY-qf and TOCSY-ph spectra were acquired with 4K data points, a spectral width of 4795.396 Hz, 2 s repetition delay, 16 dummy scans, and 32 scans for 256 series. [1]H NOESY-tppi spectra were recorded with 4K data points, spectral width of 4795.396 Hz, a mixing time of 200 ms, 2 s repetition delay, 16 dummy scans and 64 scans for 256 series.

RP-HPLC (Reverse Phase-High Performance Liquid Chromatography). This chromatographic technique enables separation, identification and quantification of substances in a mixture, as well as the detection of degradation products, combining the rapidity of execution with a high sensitivity and reproducibility.

In this work, ActrapidTM samples were run through an Agilent 1100 HPLC system, equipped with a Vydac 218TP54 column and coupled with an UV detector (230 nm), at 25°C and under isocratic conditions (mobile phase: 0.05M KH_2PO_4-CH_3CN 65:35, v/v; pH 2.4, flow rate 1.0 ml min^{-1}) [8].

MTT Assay. The *in vitro* mitogenic potency of insulin was evaluated as a functional parameter, to confirm the structural integrity of drug after RF exposure. Cell growth was monitored by measuring the ability of viable mitochondria to reduce the yellow MTT tetrazolium salt to a purple formazan product [9]. Briefly, cells were seeded into 96-well trays (4-6*10^3 cells/well) and, after 24 h incubation with 0.2% FBS hormone-free media ("serum starvation"), ActrapidTM was administered at the lowest concentration able to induce an appreciable proliferation on each cell line (5 nM). After 24 h treatment, 0.5 mg/ml MTT was added to cultures for 3 h at 37°C. The formazan product, solubilized by dimethyl sulfoxide, was measured spectrophotometrically at 570 nm (background correction at 690 nm). The proliferation of treated cultures was expressed as a percentage with respect to untreated cells, assuming the related mean absorbance value as a reference (basal growth).

E. Statistical Analysis

Results of MTT tests were expressed as mean ± standard error of three separate experiments, with four replicates at least for each treatment. Graphic and statistical elaborations were carried out by GraphPad Prism software, version 5.00 (San Diego, California, USA). Comparisons were performed by one-way ANOVA (Bonferroni post-test). Statistical significance was set at $p<0.05$ (*).

III. EXPERIMENTAL RESULTS

NMR analysis. In order to investigate potential effects of RFID systems on the structure of the drug, three lots of Actrapid (control and exposed) were analyzed by mono-(1D) and multidimensional (2D) ^{1}H NMR spectroscopy as reported in the investigation methods. Due to the presence of high concentration of excipients, such as *m*-cresol and glycerol, the NMR spectra of the insulin pharmaceutical preparation were very difficult to investigate. *m*-cresol and glycerol produce resonances overlapping with several key protein signals, such as amide and CHα protons, useful for structure interpretation. Moreover, the high concentration of *m*-cresol and glycerol did not allow us to obtain NMR spectra with a good resolution for the insulin resonances. The drug samples were, therefore, treated with diethyl ether to remove partially the excipients. After lyophilization the residue was dissolved in H_2O with 20% of deuterated acetic acid (CD_3COOD) for a total volume of 0.4 mL. The use of H_2O rather than D_2O allowed us to avoid the replacement by deuterium of labile protons, such as amide protons. CD_3COOD was used in order to lock the solvent signal and to reduce the protein self-association [10]. Moreover, CD_3COOD, in place of CD_3CN used in our previous experiments [6], was able to improve the spectra quality. On all 24 samples (i.e., control, exposed 5 min, 1, 6, and 24 h from three different lots) ^{1}H NMR water suppression and 2D NMR homonuclear ^{1}H COSY and ^{1}H TOCSY spectra were recorded. Despite generally ^{1}H NMR spectra are not very informative for protein structure, the experimental conditions (i.e., H_2O/CD_3COOD 80/20 % vol) allow to perform high resolution spectra also in the amide proton range. Interestingly, the ^{1}H NMR spectra of the control and irradiated (5 min, 1, 6, and 24 h) samples from the same lot did not show differences with regards to the amide protons (Fig. 1). These data were confirmed by 2D homonuclear spectra (^{1}H COSY and ^{1}H TOCSY). 2D ^{1}H COSY and ^{1}H TOCSY experiments give information about scalar correlations (through bonds), which involve hydrogens separated by few bonds in COSY spectra (generally 2-4, more for conjugated bonds) and gives total correlation of all protons of the chain with each other in TOCSY spectra. [1]H TOCSY spectra expansions reported in Fig. 2 show that also after 24 h of RF exposure no differences were observed between the control and irradiated samples from the same vial and lot. As reported in previous works [6], the only differences disclosed in 2D spectra were in the aliphatic region. However, these differences were present not only among the lots but also within the same lot and within both the control and irradiated samples. Therefore, the high variability of this region was not taken in account.

To avoid the limits associated to the low concentration of protein and to the presence of other substances in the pharmaceutical preparation, further NMR investigations were performed on the pure insulin molecule. The NMR samples of pure insulin, previously dissolved in H_2O/CD_3COOD 80/20 % vol, were directly irradiated by

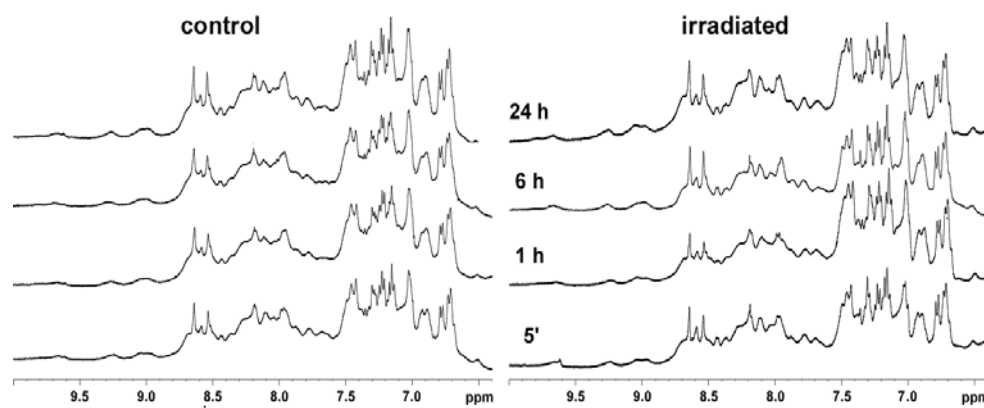Fig. 1. Expansions of ^{1}H NMR spectra in H$_2$O/CD$_3$COOD (80/20 % vol) of ActrapidTM samples from the same lot.

RFID system in the NMR tube for the same exposure times used for ActrapidTM preparation. For the pure insulin samples, the high concentration of protein and the absence of excipients, allowed to carried out, as well as ^{1}H NMR and ^{1}H COSY, also ^{1}H NOESY spectra. ^{1}H NOESY sequences are able to provide data about the hydrogens which are spatially close enough. In the case of proteins the NOESY experiments give information about the protons which are distant in the amino acid sequence but close in space due to tertiary structure. All 1D and 2D spectra of the pure protein did not exhibit alterations among the irradiated sample and the control. Interestingly, also the NOESY spectra did not show changes with specially regards to the amide region (Fig. 3).

RP-HPLC structural data. The profiles of chromatograms registered for ActrapidTM samples irradiated up to 24 h were unvaried with respect to those obtained for untreated aliquots collected from the same cartridges (data not shown). Lot-to-lot and intra-lot variability was negligible, confirming that the drug retains its own structural properties even under the longest UHF radiation (24 h).

Mitogenic activity. The structural analysis of irradiated drug aliquots did not reveal any molecular change in the active principle. A functional analysis was conducted to further confirm that following the RF exposure, the active site of insulin properly interacts with the insulin-receptor placed on the cell membranes, eliciting the expected metabolic and gene regulatory effects. A cell model suitable for cell cycle studies was employed - PCCl3, to evaluate the mitogenic potency of control, 5 min, 1, 6, 24 h irradiated ActrapidTM. The growth rate of cells cultured in 0.2% FBS media was assumed as a reference. In Fig. 4 the results of MTT assays for all three lots of drug tested are detailed. To avoid misinterpretations due to intra-lot variability, for each time exposure, regular and irradiated drug aliquots were taken from the same vial. It can be observed that the ability of insulin to stimulate cell proliferation was unvaried under UHF RF exposure. This finding was further confirmed on a breast carcinoma cell line - MCF7 (data not shown), to exclude undesirable effects (e.g. aberrant proliferation) on tumor cells. The experimental results, obtained by this technique, led to the conclusion that even a prolonged RF exposure does not interfere with the performance of ActrapidTM.
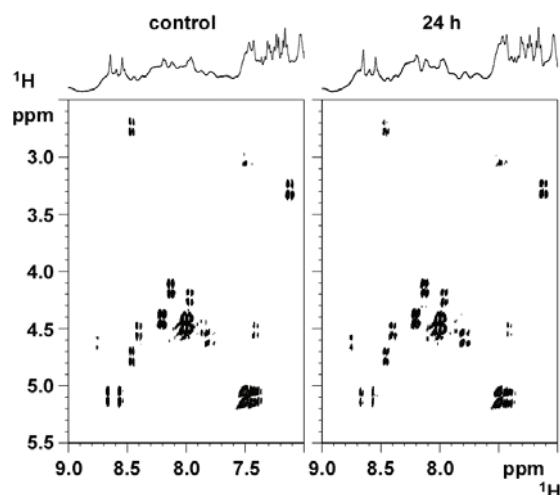

Fig. 2. Expansions of 2D ^{1}H TOCSY spectra in H$_2$O/CD$_3$COOD (80/20% vol) of ActrapidTM samples (control and irradiated) from the same vial and lot.
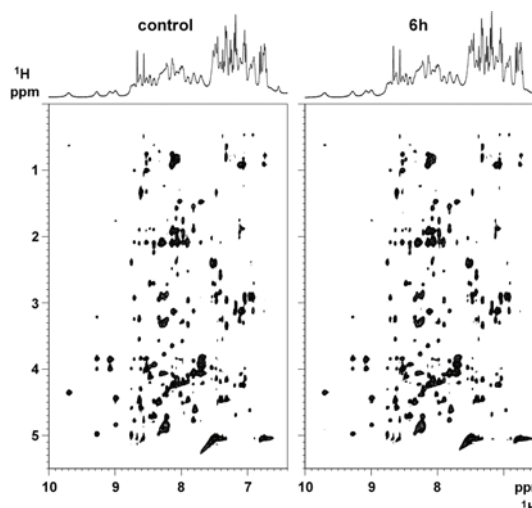

Fig. 3. Expansions of 2D ^{1}H NOESY spectra in H$_2$O/CD$_3$COOD (80/20% vol) of pure insulin (control and irradiated samples).
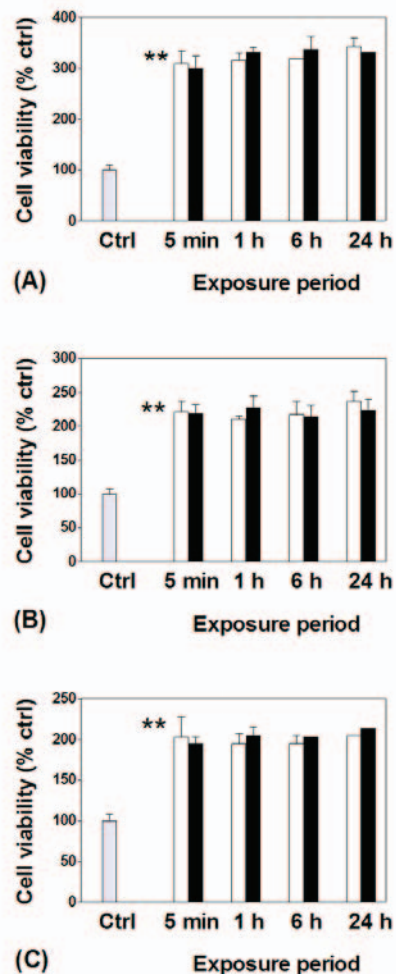
Fig. 4. Mitogenic effect of regular and UHF irradiated ActrapidTM on PCCl3 cells. Cultures were exposed to 5 nM control (*white bar*) and 5 min, 1, 6, 24 h UHF irradiated (*black bar*) drug for a day. For each exposure time, control and treated drug samples were from the same vial and separate vials were used for the indicated time period exposures. The induced growth rate was quantified respect to basal proliferation (*grey bar*). Product samples were from three production lots (nr): XS63630 (A); XS63631 (B); XS64223 (C). The proliferative potency of ActrapidTM was unaltered even after the longest time period exposure (24 h). Data points represent the mean ± st.err. of three separate experiments (**$p < 0.01$, ANOVA).

IV. Conclusion

In this work, we described an integrated analysis of the effects of Radio Frequency emissions on a commercial insulin preparation, ActrapidTM. The experimental protocol combined an accurate structural analysis on 5 min to 24 h irradiated drug samples with a functional *in vitro* assay. The NMR analysis demonstrated the molecular integrity of ActrapidTM and pure insulin after RF exposure. RP-HPLC investigations confirmed such results, being the chromatograms of treated samples superimposable on those registered from the regular product. Consistently, the ability of irradiated insulin to bind its own receptor on cell membranes was unaltered, as shown by testing its mitogenic

action on cell models. We can conclude that the effects of RF emissions on drug quality were negligible, that strongly encouraging the use of RFID-based technologies for item-level tracing systems in the pharmaceutical supply chain. In this view, it must be noticed that the sensitivity of molecules to RF may vary in dependence of their nature and steric arrangement, so a thorough analysis of the potential effects should precede the application of RFID tags to particular products. The described experimental scheme well meets this requirement, as specific variations can be introduced based on the characteristics of the compounds to be analyzed (i.e. HPLC mobile phase, targeted biological processes).

In the near future, we are extending our experimental studies to other pharmaceutical preparations, to demonstrate the goodness and usefulness of the proposed protocol.

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