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Radiosterilisation of indomethacin PLGA/PEG-derivative microspheres: Protective effects of low temperature during gamma-irradiation

Ana Fernández-Carballido *, Patricia Puebla, Rocío Herrero-Vanrell, Pilar Pastoriza

Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Complutense s/n, Universidad Complutense, 28040 Madrid, Spain

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Abstract

Currently, γ-irradiation seems to be a good method for sterilising drug delivery systems made from biodegradable polymers. The γ-irradiation of microspheres can cause several physicochemical changes in the polymeric matrix. These modifications are affected by the temperature, irradiation dose and nature of the encapsulated drug and additives. This study has aimed to evaluate the influence of temperature during the sterilisation process by gamma irradiation in indomethacin PLGA microspheres including a PEG-derivative. Microspheres were prepared by the solvent evaporation method from o/w emulsion and were then exposed to γ-irradiation. A dose of 25 kGy was used to ensure effective sterilisation. Some microspheres were sterilised with dry ice protection that guaranteed a low temperature during the process whilst others were sterilised without such dry ice protection. The effects of γ-irradiation on the characteristics of non-loaded PLGA/PEG-derivative and indomethacin loaded PLGA/PEG-derivative microspheres with and without protection were studied. Non-protected microspheres showed changes in their morphological surface, polymer glass transition temperature, molecular weight and release rate of indomethacin after sterilisation. However, microspheres sterilised with protection did not show significant differences after γ-irradiation exposure. The sterilisation method was satisfactory when the indomethacin loaded microspheres including a PEG-derivative were exposed to γ-irradiation at low temperature.

Keywords: Microspheres; PLGA; Intraarticular; PEG-derivative; Gamma irradiation

1. Introduction

Current research into the controlled delivery of pharmaceuticals involves the use of biodegradable polymers. The aliphatic polyesters based on lactic and glycolic acids have demonstrated good biocompatibility and the absence of significant toxicity (Menei et al., 1993; Rice et al., 1978) and they have been widely used to prepare injectable delivery systems such as microspheres. Pharmaceutical systems intended for parenteral administration have to meet the pharmacopoeia requirements of sterility. The chemical liability of formulation constituents limits the use of sterilisation methods to obtain an acceptable final sterile product. It is well known that a terminal sterilisation procedure is preferred over aseptic processing, because it is easier from a technological point of view. Sterilisation techniques, such as steam or dry heat cannot be used for biodegradable aliphatic polyesters such as polylactic-co-glycolic acid (PLGA) since they alter the physical and chemical properties of the biomaterial. In its turn, chemical sterilisation with ethylene oxide causes serious toxicological problems, due to residual components of the sterilising agent. Currently, γ-irradiation seems to be a good alternative for final sterilisation of drug delivery systems made from biodegradable polymers. The advantages of gamma irradiation include high penetrating power, low chemical reactivity, low measurable residues and small temperature rise. A minimum absorbed dose of 25 kGy is regarded as adequate for the purpose of sterilising pharmaceutical products without providing any biological validation (USP 28, 2005; Montanari et al., 2001).

The effects of γ-irradiation on PLGA polymers and derived formulations have been the subject of several works. For example, it has been observed that gamma irradiation can induce several physicochemical changes in the polymer such as cross-linking, chain scission, formation of radicals and a drop in the polymer molecular weight being the most reported effect (Geze et al., 2001). The decrease in the molecular weight of the PLGA

* Corresponding author. Tel.: +34 913941741; fax: +34 913941736.
E-mail address: afernand@farm.ucm.es (A. Fernández-Carballido).

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is conditioned by the temperature, irradiation dose and nature of the encapsulated drug. Generally, these structural modifications lead to a change in the release rate of the drug and they have been the subject of several studies. In fact, the release rate of clonazepam from PLGA microspheres was increased by approximately 10% after their sterilisation by gamma irradiation using a dose of 25 kGy (Montanari et al., 2001). In this case the process was carried out in air at room temperature. In another report, Yip et al. (2003) observed a drastic modification to the release profile of etanidazole microparticles made with a mixture of PLGA/PLA (PLGA M_w = 40,000; 75,000 Da and PLA M_w = 90,000; 120,000 Da) after γ irradiation (25 kGy) at low temperature (−78 °C). Nevertheless, the same irradiation dose (25 kGy) did not produce changes in low molecular weight PLGA microspheres. In this experiments by Yip et al. a low temperature was maintained during the whole irradiation process, by protecting the samples with dry ice. Nevertheless, under these previous conditions no significant differences were observed between the release profiles of sterilised and non-sterilised PLGA microspheres loaded with ganciclovir (PLGA M_w = 34,000 Da) and aciclovir (PLGA M_w = 12,000 Da) (Herrero-Vanrell et al., 2000; Martinez-Sancho et al., 2004). In both cases, the drug was incorporated in the microspheres as a suspension and the initial properties of the particles were maintained. Similar results were obtained for ibuprofen PLGA microspheres (M_w = 12,000 Da). In this case, the drug was practically dissolved in the polymeric matrix (Fernández-Carballido et al., 2004a), demonstrating that the conditions employed were suitable for low molecular weight PLGA microspheres including drugs which are at different physical states in the polymeric matrix. Taking into account the fact that the same irradiation dose was employed in all cases, the results mentioned above can be attributed to the use of low molecular weight PLGA polymers and low temperature during the process.

The effect of γ-irradiation on the PLGA molecular weight is also influenced by the nature of the drug but it is not easily predicted due to the varying chemico-physical characteristics of the active substance and its interaction with the polymer. In some cases the drop in polymer molecular weight occurred independent of the active molecule included in the formulation. However, in other studies the entrapped drug accelerated polymer degradation usually when the drug is an acidic substance which could act as catalyst agent (Lin et al., 2000; Li et al., 1996; O'Donell and Mcginity, 1998).

Another important point relates to the presence of additives in the formulation. These kinds of agents have been shown to modify the initial characteristics of microspheres such as morphology, drug encapsulation efficiency and in vitro release of the active substance (Hedberg et al., 2002; Lee et al., 2002a,b). Also, alterations in the glass transition temperature (T_g) of the polymers have been observed. Taking into account that a great number of these agents are oils, they may be altered by temperature changes.

Indomethacin (IM, γ-indomethacin; 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid) is an acidic drug with analgesic and anti-inflammatory properties. This non-steroidal agent is sparingly soluble in water and can exist in several crystalline forms but generally, it has been used in pharmaceutical preparations as Form I (type γ). IM has been reported to present physicochemical interactions with several substances (Watanabe et al., 2001).

In this experiment indomethacin biodegradable microspheres have been prepared using a PEG-derivative (Labrafil®) as an additive. The polymer employed has been PLGA 50:50 RG®503 (M_w = 34,000 Da) and oil was used to extend the release time of the drug (Fernández-Carballido et al., 2004b).

Until now, there has been no evidence about the effect of protecting the samples from a rise in temperature, during gamma irradiation exposure in formulations including additives. This study has therefore aimed to evaluate the sterilisation process by gamma irradiation in indomethacin PLGA microspheres including a PEG-derivative. The influence of temperature on the final properties of the formulations was studied. For this purpose, microspheres were exposed to a effective sterilising dose (25 kGy) with and without protection. Samples were protected with dry ice, ensuring a low temperature during the process.

2. Materials and methods

Indomethacin (γ or I form) was supplied by Sigma–Aldrich Chemical (Madrid, Spain). PLGA 50:50 poly(D,L-lactide-co-glycolide) Resomer® RG 503 [M_w = 34,000 Da (GPC)] was purchased from Boehringer Ingelheim (Ingelheim, Germany). PEG- derivative, Labrafil® M 1944 CS was supplied by Cattefossé (Saint-Priest, France). Polyvinyl alcohol (PVA) [M_w = 49,000 Da] was supplied from Sigma–Aldrich Chemical (Madrid, Spain). Dichloromethane and methanol, analytical grade were provided by Merck (Darmstadt, Germany). Distilled and deionized water (Millipore Corporation, Bedford, MA, USA) was used in the preparation of all buffers and solutions.

2.1. Preparation of microspheres

Microspheres were prepared by the solvent evaporation method based on an oil-in-water (o/w) emulsion. The polymer PLGA (200 mg), indomethacin (40 mg) and PEG-derivative (20 μl) were dissolved in 1 ml CH_2Cl_2. The oil was added in the inner phase of emulsion. The external phase of the emulsion was a solution of 1% PVA in distilled water. Once prepared the organic phase was poured into 5 ml of the aqueous phase. The resulting emulsion was stirred using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) at a speed setting of 2000 rpm for 2 min. Upon formation of the emulsion 6 ml of distilled water was added and stirred continuously at the same speed setting for 1 min. Then, the immature microspheres were suspended in 250 ml of distilled water and the system was agitated for 4 h at room temperature, to allow complete evaporation of the organic solvent. Finally, the microspheres were filtered under vacuum using a 5 μm filter and placed in a vacuum desiccator at 25 °C for at least 48 h.

Non-loaded microspheres including the PEG-derivative were also prepared using the technique described above.

2.2. Gamma-irradiation of indomethacin microspheres

Polymer, non-loaded microspheres and indomethacin loaded microspheres were placed in 5 ml glass vials. The samples were then packed in dry ice inside a polyurethane container as described previously (Herrero-Vanrell et al., 2000). The conditions employed ensured a low temperature during the sterilisation procedure. Raw polymer, non-loaded microspheres and indomethacin loaded microspheres were also sterilised without dry ice. All samples were irradiated using ^{60}Co as the radiation source in the γ-radiation unit at Aragogamma S.A. (Barcelona, Spain). In accordance with European Pharmacopeia recommendations, a γ-radiation dose of 25 kGy was used to ensure effective sterilisation. The formulation was analysed before and after the sterilisation process.

Assays were carried out in triplicate for each of the five batches of microspheres used for this work.

2.3. Morphological characterisation and size distribution

Scanning electron microscopy (JEOL, JSM 6400, Tokyo, Japan) was used to study the surface morphology of the microspheres before and after the sterilisation process. Before observation by SEM at 20 kV, non-sterilised and sterilised samples, with and without protection, were coated with a thin layer of colloidal gold applied in a cathodic vacuum evaporator.

Granulometric analysis (mean diameter and size distribution) of each microsphere batch was performed by laser diffraction using a Galai model Cis-1 computerized inspection system (Migdal Haemek, Israel) within the 0.5–150 μm range. Samples of microspheres were suspended in distilled water and analysed for particle size whilst being gently stirred. Results are expressed as volume-density mean diameter.

2.4. Determination of indomethacin encapsulation efficiency

A fixed weight of microspheres (20 mg) was first dissolved in 1 ml of dichloromethane. Then, the polymer was precipitated with acetonitrile (4 ml). The mixture was then centrifugated at 5000 rpm for 10 min (Eba 12R centrifuge, Hettich, Germany) and the supernatants were filtered through 0.45 μm filters (PTFE filter, Tracer Spain). The indomethacin content in the PLGA/PEG-derivative microspheres was determined by HPLC. The mobile phase consisted of buffer phosphate (0.01 M) and acetonitrile (63/37) the wavelength was set at 254 nm. The column employed was a Lichrospher 100 RP 18, 5 μm (25 cm × 0.4 cm). Under these conditions, the retention time of indomethacin was 5.05 min.

Encapsulation efficiency was calculated as the ratio of the encapsulated drug referred to the initial amount of the drug used for the microsphere preparation. Samples were taken and the results studied from both before and after sterilisation (with and without dry ice protection).

2.5. X-ray diffraction analysis

X-ray diffraction patterns were obtained using a Philips X'Pert model MPD diffractometer (Almedo, The Netherlands) with a Cu Kα radiation, θ–2θ powder diffractometer set for an angle range of (5–50°)/2θ, step size of 0.04° 2θ, and count times of 1 s per step. The indomethacin, polymer, non-loaded microspheres and indomethacin loaded microspheres were scanned under these settings. Samples were analysed before and after their exposure to γ-radiation with and without dry ice.

2.6. Differential scanning calorimetry (DSC)

The physical state of the microspheres compounds were determined by thermal analysis. The analyses were performed using a Mettler model DSC TA8000 calorimeter (Greinfensee, Switzerland) using nitrogen as a purging gas. The samples (10 mg) were transferred to and sealed in aluminium pans. An empty aluminium pan was used as a reference. The temperature range tested was from 20 to 200 °C and the heating rate was 10 °C/min. Under these conditions, DSC thermograms were undertaken for indomethacin, polymer, non-loaded microspheres and indomethacin loaded microspheres. Samples were analysed before and after their exposure to γ-radiation with and without dry ice.

2.7. Gel permeation chromatography (GPC)

Molecular weights are expressed as weight-average molecular weight (M_w) and number-average molecular weights (M_n). Microparticles were dissolved in tetrahydrofuran (THF) 10 mg/ml. After filtration (PTFE filter, pore size 0.45 μm, Tracer Spain), 20 μl of the solution was injected. Two columns HR 4 E column (7.8 mm × 300 mm, Waters Associates, MA, USA) and Ultrastygel 103 Å (Waters Associates, MA, USA) were connected consecutively to increase the accuracy of the procedure. All measurements were taken at a flow rate of 1 ml/min at 35 °C with a Waters 1525 binary HPLC pump. The refractive indexes were measured using a 2414 refractive index detector (Waters Associates, MA, USA). Molecular weight was calculated by the system calibration software using narrow polystyrene reference materials of known molecular weights: 114.0, 43.7, 18.6, 9.6, 6.5 and 2.9 kDa (Waters Corporation, Polymers Standard Service GmbH, Germany). Evaluation was done according to a cubic universal calibration curve (Waters, MA, USA). Samples were analysed before and after their exposure to γ-radiation with and without dry ice.

2.8. In vitro release study

Sterilised and non-sterilised indomethacin loaded microspheres (20 mg) were suspended in 4 ml of isotonic phosphate buffer saline (PBS), pH 7.4 (sink conditions) and placed in a water shaker bath (model NE-28, Clifton, U.K.) at 37 °C with constant agitation (50 strokes/min). At regular time intervals, the PBS was removed with a syringe, filtered through a 0.45 μm

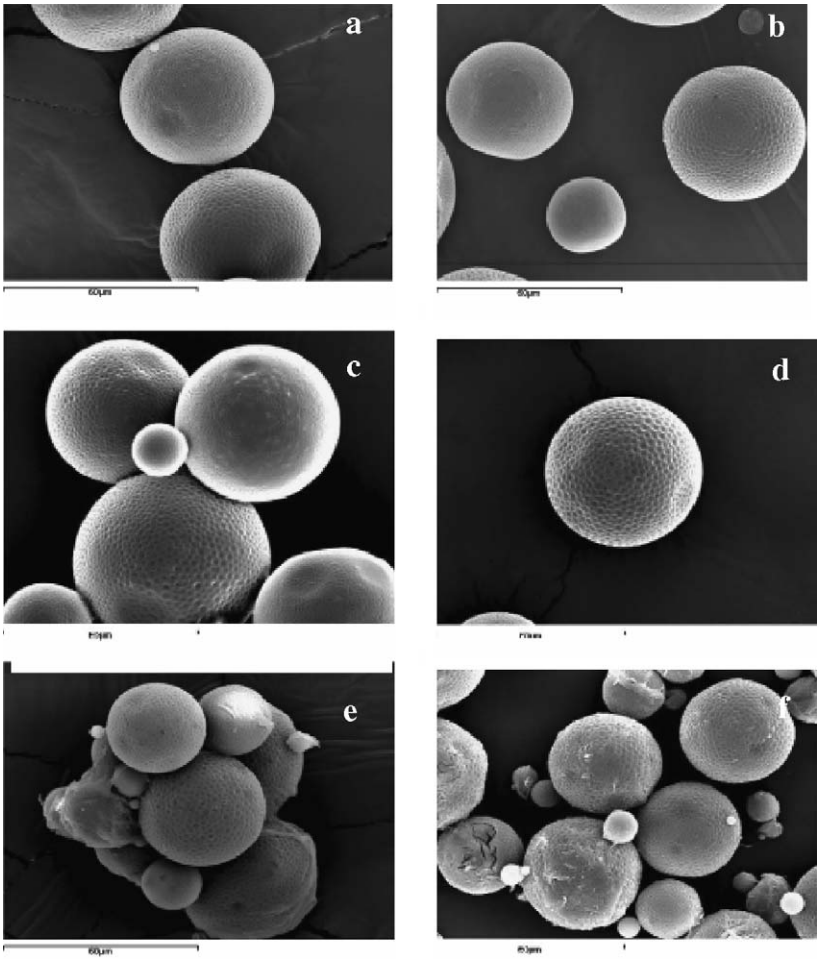

Fig. 1. Electron micrographs of non-loaded microspheres (a) and indomethacin-loaded microspheres (b) before sterilisation; non-loaded microspheres sterilised with dry ice protection (c); indomethacin-loaded microspheres sterilised with dry ice protection (d); non-loaded microspheres sterilised without dry ice protection (e); indomethacin-loaded microspheres sterilised without dry ice protection (f) (300× magnification).

filter (Teknokroma) and indomethacin concentration was determined spectrophotometrically at 318 nm. The components of the microspheres did not interfere with indomethacin at this wavelength. The same volume of fresh medium was replaced to continue the release study. The release test was performed in triplicate for each batch of microspheres (five batches).

3. Results and discussion

The aim of this work was to study the influence of temperature, during the sterilisation process by γ-irradiation, on the final characteristics of indomethacin PLGA/PEG-derivative microspheres.

Five different batches of microspheres in a ratio 2:1:10 (indomethacin:PEG-derivative:polymer) were weighed (20 mg) and transferred to 5 ml glass aluminium sealed cap vials. After labelling, the vials were sterilised with and without protection during the irradiation process. Samples of polymer, indomethacin and non-loaded microspheres were sterilised in the same conditions employed for the indomethacin loaded microspheres.

3.1. Microsphere morphology

Morphologically, SEM revealed that non-sterilised indomethacin microspheres with PLGA/PEG-derivative possessed a homogeneous shape, showing small concavities on their surface (Fig. 1). Morphology of protected microparticles was not affected by irradiation exposure. On the contrary, non-protected microspheres appeared aggregated after sterilisation (Hausberger et al., 1995). In the present study, the morphological changes observed in the non-protected samples indicated that the temperature rise led to particle fusion. Similar results have been previously described by other authors (Montanari et al., 1998). Furthermore, the increase in temperature modified the characteristics of the polymeric matrix as the broken surface of the non-protected microparticles and the additive (PEG-derivative) was escaping from the particles.

Indomethacin microspheres before sterilisation showed a homogeneous size distribution with a mean size of around 50 μm. This size of microparticle can be considered suitable for intraarticular administration because it can be injected through a syringe attached to a 27G needle. The size distribution of indomethacin microspheres (46.6 ± 3.5 μm) was not affected

Table 1
Values of glass transition temperature (T_g) for polymer, non-loaded microspheres and indomethacin loaded microspheres before and after γ-irradiation

	T_g before sterilisation (°C)	T_g sterilisation without dry ice protection (°C)	T_g sterilisation with dry ice protection (°C)
Polymer (PLGA 503)	44.57 ± 0.4	43.05 ± 0.8	42.43 ± 0.7
Non-loaded microspheres	41.51 ± 0.5	39.14 ± 0.4	41.06 ± 0.6
Indomethacin loaded microspheres	40.30 ± 0.2	35.28 ± 0.5	40.39 ± 0.6

The results are expressed in °C.

by the sterilisation process when the samples were surrounded by dry ice during irradiation exposure (48.1 ± 4.2 μm). Nevertheless, if the vials were not protected the mean diameter of the particles resulted increased (59.2 ± 5.5 μm). This phenomenon can be ascribed to the presence of aggregates observed by SEM.

3.2. Indomethacin encapsulation efficiency

The indomethacin loading efficiency for the non-sterilised microspheres was 94.6 ± 0.1%. The encapsulation efficiency values were lower for the sterilised formulations, being 92.2 ± 0.2% and 90.5 ± 0.2% for protected and non-protected microparticles, respectively. The loss of indomethacin in the microspheres could be attributed to the degradation of the drug caused by the irradiation. In fact, the loss of indomethacin was highlighted after the sterilisation of the drug alone (data not reported), showed a higher loss of indomethacin than the results observed for indomethacin included in the microspheres. Indomethacin contains polar groups able to form hydrogen bonds with the PLGA polymer. In this test, the interaction between IM and polymer probably brought about a decrease in IM molecular mobility and hence increased drug stability.

3.3. Differential scanning calorimetric assays

The physical state of the components of microspheres compounds was determined by thermal analysis. Samples of polymer, indomethacin, non-loaded microspheres and indomethacin-loaded microspheres were tested. The non-loaded microspheres exhibited a lower glass transition temperature (T_g = 41.51 °C) than the PLGA raw material (44.57 °C), probably due to a PEG-derivative plasticizer effect (Blanco-Prieto et al., 2000; Choi et al., 2001). As shown in Table 1 γ-irradiation caused only a slight decrease in the T_g values of the polymer and non-loaded microspheres with and without protection with dry ice. This was not the case for indomethacin-loaded microspheres in which the peak of the drug was practically absent (indomethacin showed a characteristic melting point at around 162 °C, corresponding to form I) and the T_g value due to polymer was 40.30 °C (Watanabe et al., 2001; Lin et al., 1999). When comparing the DSC thermograms of sterilised and non-sterilised indomethacin microspheres it was clearly observed that temperature was an influence. In fact, the T_g value for the non-protected samples was significantly lower (35.28 °C) than the one observed for the indomethacin microspheres surrounded with dry ice during the irradiation (40.39 °C).

3.4. X-ray diffraction analysis

The results of X-ray diffraction analysis of raw polymer (RG®503) showed no maximums confirming its amorphous state (Fig. 2). The microencapsulation process did not modify the polymer characteristics as could be observed in the X-ray diffraction patterns of non-loaded microspheres. The highest levels of intensity in the X-ray diffraction pattern for indomethacin were obtained at 11.60°, 16.65°, 17.00°, 19.30°, 19.60°, 21.80°, 26.62°, 28.9° and 29.35°. Nevertheless, loaded microspheres showed an irregular baseline typical pattern of an amorphous state without the strongest maxima of indomethacin, suggesting a loss of crystallinity of the drug inside the microspheres. This fact was also observed in sterile indomethacin microspheres after γ-irradiation.

3.5. Gel permeation chromatography (GPC)

Average molecular weight (M_w) is the most relevant parameter to evaluate polymer degradation. In order to establish the influence of temperature during sterilisation, the weight-average molecular weight (M_w) was evaluated by GPC (Table 2). Before sterilisation, the M_w for the RG 503 polymer was in 35.0 ± 2.9 kDa. In its turn, the weight average molecular weights for the non-loaded microspheres and indomethacin

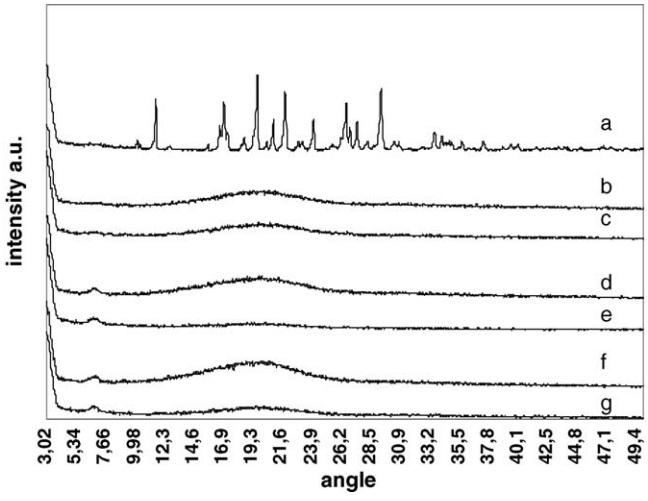

Fig. 2. X-ray powder diffraction patterns of: Indomethacin (a); non-loaded microspheres (b); indomethacin-loaded microspheres (c); non-loaded microspheres sterilised with dry ice protection (d); indomethacin-loaded microspheres sterilised with dry ice protection (e); non-loaded microspheres sterilised without dry ice protection (f); indomethacin-loaded microspheres sterilised without dry ice protection (g).

Table 2
Mean molecular weight (M_w) and number-average molecular weights (M_n) values for non-loaded microspheres and indomethacin loaded microspheres before and after γ-irradiation

Formulation	Mean molecular weight (kDa)
Non-loaded microspheres before sterilisation	36.0 ± 2.2
Non-loaded ME with dry ice protection	35.8 ± 2.0
Non-loaded ME without dry ice protection	34.4 ± 3.3
Indomethacin loaded ME before sterilisation	34.6 ± 1.7
Indomethacin loaded ME sterile with dry ice protection	32.2 ± 3.3
Indomethacin loaded ME sterile without dry ice protection	29.2 ± 5.0

loaded microspheres were close, demonstrating that the solvent evaporation process and the indomethacin addition did not influence the polymer M_w. Microspheres loaded with indomethacin showed a decrease in M_w after irradiation. For non-protected microparticles the reduction in M_w was about 15.6% whilst for the protected ones the reduction was only 6.9%. These results were in agreement with the ones observed in DSC studies. The impact of γ-irradiation in low molecular weight PLGA at room temperature has been already associated with an increase in macromolecular chain polydispersity (Geze et al., 2001; Faisant et al., 2002; Lee et al., 2002a,b). In the reported study, the influence of temperature was also demonstrated, showing a higher decrease of M_w for the samples sterilised without dry ice protection.

When the drug was present in the polymer matrix the M_w values were modified in both the non-protected and protected samples. The higher reduction of M_w was observed for the samples sterilised without dry ice protection. Nevertheless, due to the high standard deviations obtained for the M_w values non-significant differences were obtained between the non-sterilised and sterilised microspheres with and without protection for this parameter ($p = 0.1216$ for indomethacin loaded microspheres and $p = 0.5932$ for non-loaded microspheres).

3.6. In vitro release study

Indomethacin release from microspheres in PBS pH 7.4 before and after γ-sterilisation (protected and non-protected) are shown in Fig. 3.

The release pattern from indomethacin microspheres before γ-irradiation exhibited a near zero-order kinetic for 21 days. The initial burst (8 h) for non-sterilised microspheres was 9.4 ± 1.4%, with no significant alteration ($p > 0.05$) after γ-radiation exposure for protected microparticles (13.8 ± 2.5%). The release profiles of indomethacin microspheres after γ-irradiation with dry ice protection showed a profile similar to the one observed for the non-sterilised microspheres. This was not the case for microspheres sterilised without protection where there was an initial burst of about 17.4 ± 3.8% and a release rate of indomethacin faster than the one observed before their sterilisation. This result was mainly attributed to additives outside the polymer matrix in the non-protected microspheres as observed by SEM.

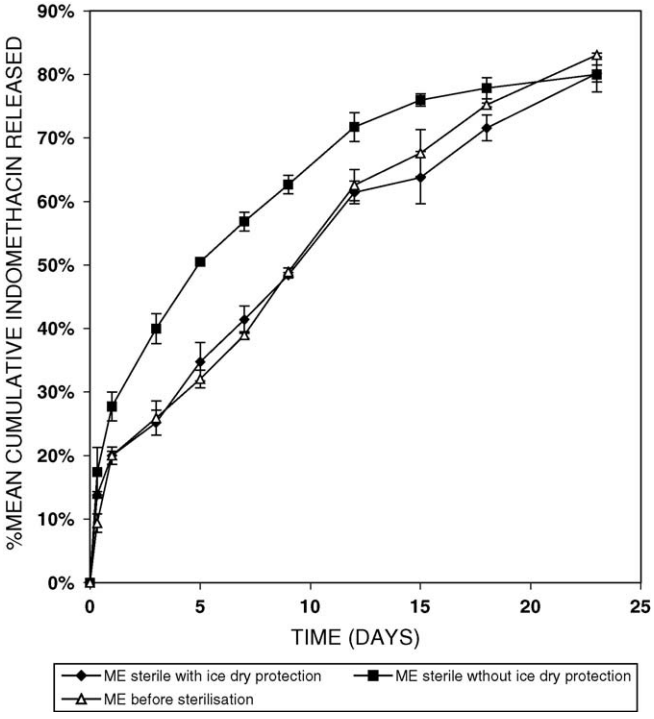

Fig. 3. Mean cumulative percentages of indomethacin release (±S.D.) from 20 mg of microspheres before and after γ-irradiation.

Table 3
f_1 and f_2 values for sterilized microspheres with and without dry ice protection

	Indomethacin loaded ME sterile with dry ice protection	Indomethacin loaded ME sterile without dry ice protection
Difference factor (f_1)	11.58	18.74
Similarity factor (f_2)	76.23	45.60

Difference (f_1) and similarity (f_2) factors were calculated to compare indomethacin release profiles from microspheres before and after sterilisation (Table 3). Both factors allow determination of dissolution profiles (i.e. it was estimated that a value for the similarity factor ranging between 50 and 100 indicated similarity between curves) (Martinez-Sancho et al., 2004; Fernández-Carballido et al., 2004a). The values were satisfactory only when the samples were protected with ice and indicated that the curves were similar and overlapping. In this case, the protection with dry ice avoided changes in the indomethacin release rate from PLGA microspheres.

4. Conclusions

The sterilisation process reported in this study was suitable for indomethacin PLGA/PEG-derivative microspheres in the samples protected from a temperature rise with dry ice. Although a decay in the M_w of polymer was observed under these conditions, the release rate of indomethacin was similar

for both sterilised and non-sterilised microspheres at low temperature. The results observed in this study showed the effectiveness of protecting low molecular weight PLGA including additives from the rise of temperature during γ-irradiation exposure.

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