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Research Article

Stability Evaluation of Ivermectin-Loaded Biodegradable Microspheres

Rossella Dorati,[1] Ida Genta,[1] Barbara Colzani,[1] Tiziana Modena,[1] Giovanna Bruni,[2] Giuseppe Tripodo,[1] and Bice Conti[1,3]

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Abstract. A stability study was performed on ivermectin (IVM)-loaded biodegradable microparticles intended for injection in dogs. The rational was to evaluate the performances upon irradiation of a drug, such as IVM, with a few criticalities with respect to its stability, and toxicity. The goal was to provide valuable information for pharmaceutical scientists and manufacturers working in the veterinary area. The microspheres based on poly(D,L-lactide) and poly-(ε-caprolactone) and loaded with IVM and with the addition of alpha-tocopherol (TCP) as antioxidant were prepared by the emulsion solvent evaporation method and sterilized by gamma irradiation. Microsphere characterization in term of size, shape, polymer, and IVM stability upon irradiation was performed. The results show that the type of polymer significantly affects microsphere characteristics and performances. Moreover, suitably stable formulations can be achieved only by TCP addition.

KEY WORDS: alpha-tocopherol; gamma irradiation; ivermectin; microspheres; poly(D,L-lactide); poly-(ε-caprolactone).

INTRODUCTION

Ivermectin (IVM) provides potent "safety-net" activity against *Dirofilaria immitis*. For this reason, the drug is on the market in several pharmaceutical veterinary products to be administered in dogs or cats preferentially as oral dosage forms. The development of a safe injectable drug delivery system providing a 6-month or 1-year extended coverage of antiparasitic effect is highly envisaged for the animal owners convenience, in order to avoid the monthly administrations. However, IVM characteristics, a BCS class II drug with low aqueous solubility and high permeability, make it difficult to set it up. At the moment, the only injectable product on the market is a suspension that is not always recommended by veterinarians for small tale animals because of its side effects that are likely to be caused by the uncontrolled IVM plasma levels reached. As reported in a previous work of the research group (1), injectable biodegradable microparticles based on polymers such as poly (DL-lactide) and poly-(ε-caprolactone) could be a suitable drug delivery system. They would provide more control on IVM plasma levels over extended time and enable significant improvement in IVM administration compliance, skipping the problems raising from animal owner compliance failure. In the literature, it can be found in a recent study by Camargo *et al.* (2) that evaluates an *in situ* forming polylactide-based implant for the sustained release of IVM. On the same purpose, a preliminary work has been published by the research group on the preparation and characterization of Ivermectin-loaded biodegradable microparticles based on poly (DL-Lactide) and poly-(ε-caprolactone) (1). Microparticulate drug delivery systems have the advantage to be easily injected subcutaneously as suspension with a regular intramuscular (i.m.) syringe needle (21 gauge). Moreover, the formulation, whenever freeze dried, is a powder easy to be stored and suspended in a suitable aqueous solvent. However, IVM chemical structure makes it sensitive to oxidation, particularly upon the sterilization processes commonly used in the pharmaceutical industry, thus rendering tricking the achievement of a sterile stable product.

Since it has been proved that ionizing irradiation is the most suitable sterilization process for moisture and heat sensitive polymers such as poly (DL-lactide) and poly-(ε-caprolactone) (3–6), the process has been chosen for IVM-loaded microparticles sterilization and the goal of the present paper is to evaluate the effects of the sterilization process on the IVM-loaded microparticles, in terms of IVM and polymer stability, and the drug delivery system performances. It is known that this process can lead to remarkable alterations in the polymer materials during, immediately after, or even days, weeks, or months after irradiations (4–7). The extent of these alterations depends on the chemical composition, the structure of polymer and the drug, the total radiation dose absorbed, and the rate at which the dose was deposited. Alteration extent is also affected by the environmental conditions under which the radiation treatment was carried out, and the post radiation storage environment (7–13). The presence of oxygen or air during irradiation produces free radicals that

[1] Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100, Pavia, Italy.
[2] Department of Chemistry, section of Physical Chemistry, University of Pavia, Viale Taramelli 12, 27100, Pavia, Italy.
[3] To whom correspondence should be addressed. (e-mail: bice.conti@unipv.it)

are often rapidly converted to peroxidic radicals. The fate of these radicals depends on the nature of the irradiated polymer and drug, the presence of additives, and other parameters such as temperature, total dose, dose rate, and sample size. In case of poly-alpha-hydroxyacids, the polymer radicals generated by gamma-irradiation transform into oxidized moieties if there is oxygen in proximity of the formed radicals that can be also trapped in the polymer matrix for a certain period of time after irradiation. These trapped radicals may further undergo some reactions during storage time after irradiation, resulting in significant alterations of the physical properties of the irradiated polymer (4–15).

For these reasons, a sterilization process should be set up and evaluated on purpose for the product, and in the present case, following the preliminary formulation studies (1) alpha-tocopherol was added to the microparticle formulation as the antioxidant excipient, to overcome the cited instability issues. The microparticles were prepared by the emulsification solvent evaporation method, the sterilization process by gamma irradiation was performed at 25 kGy dose, the conditions suggested by current E.U. Pharmacopeia and that enable no biological process validation (3). A 6-month stability study was performed storing the irradiated lyophilized microparticles in sealed glass vials at 4°C in refrigerator.

MATERIALS AND METHODS

Materials

Poly (DL-lactide) (100 DL 7E) 100% lactide, inherent viscosity 0.78 dL/g for 0.5%*w/v* in chloroform, Tg 49.5°C; poly-(ε-caprolactone) 100 CL 10E, 100% ε-caprolactone, inherent viscosity 0.80–1.20 dL/g for 0.5%*w/v* in chloroform, Tg≅ −60°C; poly(ε-caprolactone) 100 CL 19E 100% ε-caprolactone, inherent viscosity 2.06 dL/g for 0.5%*w/v* in chloroform, Tg≅−60°C, and poly(ε-caprolactone) 100 CL 20.4E, 100% ε-caprolactone, inherent viscosity 2.04 dL/g for 0.5%*w/v* in chloroform), Tg≅−60°C, were purchased from Lakeshore Biomaterials™—SurModics Pharmaceuticals (Birmingham, AL, USA). Ivermectin (IVM) PhEur (IVM) was kindly supplied by Ceva Santé Animal (Libourne, France). The solvents used in the preparation of microparticles (methylene chloride) and for HPLC analysis (methanol and acetonitrile) were purchased from Carlo Erba, Milan (Italy). Alpha tocopherol (TCP) and Polyvinyl alcohol (PVA, 87–89% hydrolyzed, M_w of 85–146 kDa) were purchased from Sigma-Aldrich, Milan (Italy). Unless specified, all other solvents and reagents were of analytical grade.

Methods

Microspheres Preparation

The microspheres were prepared following the emulsion solvent evaporation method. As known, the technique is based on the emulsification of an organic solution of polymer in a water based solution and the subsequent evaporation the polymer organic solvent. The microsphere composition and preparation process were set up in a previous preliminary work of the research group (1) according to the different physical-chemical characteristics of the polymers DL7E, CL10E, CL19E, and CL 20.4E. Since IVM is a radiation sensitive drug, TCP was added as antioxidant in a previously defined suitable percentage (1).

Briefly, microsphere preparation was performed as follows: 150 mg of the polymers were dissolved in 10 ml of methylene chloride (1.5%*w/v*) under magnetic stirring (300 rpm) at iced bath temperature. IVM (polymer: IVM ratio 1.5:1 *w/w*) and TCP (1%*w/v*) were dissolved in the polymer solution. The polymer-IVM/TCP solution was dropped into 160 ml of 2% *w/w* PVA aqueous solution and emulsified under magnetic stirring at 700 rpm and 15°C. When working with 100 DL 7E polymer, the emulsion temperature was gradually risen to 37°C to promote solvent evaporation, and this temperature was kept for 6 h. Since the glass transition temperature (Tg) of poly(ε-caprolactone) polymers (100 CL 10E, 100 CL 19E and 100 CL 20.4E) are between −50°C and −60°C and makes the microparticles sticky and unstable at 37°C, the solvent evaporation working conditions used for the poly(ε-caprolactone) polymers were as follows: 7 h at 23°C under mild stirring achieved by a Eurostar digital apparatus equipped with paddle (IKA labortechnik, Germany).

Afterwards, the microparticle suspensions were centrifuged for 10 min at 3000 rpm (Heraeus Christ, HiTech Trader, NJ), washed with bidistilled water (10 ml for three times) and filtered with 0.45-μm filter (Millipore Corporation, Massachussets, USA). The batches were freeze dried at −50°C, 0.01 bar for 72 h (Lio 5P, Cinquepascal s.r.l., MI, Italy), and then, they were stored in sealed brown glass vials at 4°C.

The microsphere composition is detailed in Table I. Each microsphere composition was prepared in triplicate.

Microspheres Sterilization by Ionizing Irradiation

The microspheres were treated by gamma irradiation at 25 kGy dose and 1.6 kGy/h dose rate. Irradiation was performed at the Applied Nuclear Energy Laboratory (L.E.N.A.) of the University of Pavia using ^{60}Co as the irradiation source. Twenty-five kilogray represents the minimum absorbed dose considered adequate for the purpose of sterilizing pharmaceutical products without providing any biological validation (3,13,16). Lower doses can be used only if a validation study has been carried out, while irradiation doses higher than 25 kGy may be used for research purposes to exasperate the phenomena that can be generated by irradiation such as peroxidic radical formation, oxidation, chain scission, or cross linking.

Samples irradiation was performed in brown vials and under vacuum to limit the damages induced by the interaction of oxygen molecules with radicals formed during the irradiation sterilization. Alanine dosimeters were used as dose control systems while a thermometric control was applied to check that sample temperature did not significantly increase (±1°C) above the room temperature during the irradiation.

Stability Study

A 6-month stability study in regular storage conditions was performed on the irradiated and not irradiated microspheres. Placebo and IVM-loaded microspheres batches with or without TCP were evaluated for each polymer composition.

Table I. Microsphere Composition

Batches #	Polymer			IVM:pol ratio (*w*:*w*)	IVM theoretical content *w*/*w*%	TCP *w*/*w*%
	Identification	Intrinsic viscosity (dL/g)	Molecular weight (kDa)			
PL1[a]	100DL7E, poly (DL-lactide)	0.78	124	–	–	–
IVM1[b]	100DL7E, poly (DL-lactide)	0.78	124	1:1.5	40	–
$PL1_{TCP}$	100DL7E, poly (DL-lactide)	0.78	124	–		1
$IVM1_{TCP}$	100DL7E, poly (DL-lactide)	0.78	124	1:1.5		1
PL2	100 CL10E, poly-(ε-caprolactone)	0.80–1.20	125	–	–	–
IVM2	100 CL10E, poly-(ε-caprolactone)	0.80–1.20	125	1:1.5	40	–
$PL2_{TCP}$	100 CL10E, poly-(ε-caprolactone)	0.80–1.20	125	–	–	1
$IVM2_{TCP}$	100 CL10E, poly-(ε-caprolactone)	0.80–1.20	125	1:1.5	40	1
PL3	100 CL20.4E, poly-(ε-caprolactone)	2.04	210	–	–	–
IVM3	100 CL20.4E, poly-(ε-caprolactone)	2.04	210	1:1.5	40	–
$PL3_{TCP}$	100 CL20.4E, poly-(ε-caprolactone)	2.04	210	–	–	1
$IVM3_{TCP}$	100 CL20.4E, poly-(ε-caprolactone)	2.04	210	1:1.5	40	1
PL4	100 CL19E, poly-(ε-caprolactone)	2.06	274	–	–	–
IVM4	100 CL19E, poly-(ε-caprolactone)	2.06	274	1:1.5	40	–
$PL4_{TCP}$	100 CL19E, poly-(ε-caprolactone)	2.06	274	–	–	1
$IVM4_{TCP}$	100 CL19E, poly-(ε-caprolactone)	2.06	274	1:1.5	40	1

[a] PL states for placebo microparticles
[b] IVM states for IVM-loaded microparticles

All samples were stored in vacuum-sealed brown vials, at 4°C in refrigerator. The stability parameters tested by gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC), were, respectively, as follows: polymer weight average molecular weight (M_w), polymer average molecular number (M_n), polymer polydispersity index (PI), and the IVM encapsulation efficiency and its molecular changes upon irradiation. The samples were HPLC and GPC tested at time 0, after 3 and 6 months (end of the stability study) storage. The results are reported for each batch in terms of encapsulation efficiency, percentages of the polymers M_w, M_n, and IP variations with respect to the not irradiated corresponding batches, and they are always the averages of three samples for each batch, as calculated by equations (1, 2 reported in the "Gel Permeation Chromatography Analysis" section). *In vitro* release tests were performed on the microspheres batches immediately after preparation and gamma irradiation (time 0) and after 6 months storage at 4°C (time 6), not irradiated microspheres were always tested as control. *In vitro* release test lasted 6 months, or until the drug release was completed. Moreover, particle size and morphology was analyzed at the times stated in the stability study protocol (0, 3, 6 months).

Microsphere Characterization

As stated here above microsphere characterization was focused to evaluate the effects induced by gamma irradiation on the microsphere polymer matrix and the drug, and on their stability. The characterization methods were applied as follows.

Particle Size and Morphological Analyses

Particle size distribution was determined on the microsphere samples before and after irradiation treatment. The analyses were performed by laser diffractometry using a Mastersizer (Malvern Instruments, Malvern, UK). The mean particle size (d_{10}, d_{50}, and d_{90}), the polydispersity index (SPAN), the uniformity, and the weighted residual were calculated automatically using the software provided. Three analyses were performed for each sample, and each sample was recorded ten times.

The microsphere morphology was analyzed by scanning electron microscopy (SEM). The samples were placed on sample holders and gold sputtered under vacuum; micrographs were collected with a Zeiss EVO MA10 (Carl Zeiss, Oberkochen, Germany) electron microscopy working at high vacuum.

Gel Permeation Chromatography Analysis

Gel permeation chromatography (GPC) permits to evaluate the effects of irradiation treatment on the polymer M_w, M_n, and PI. A GPC system consisting of three Ultrastyragel columns connected in series (7.7×250 mm each, with different pore diameters: 10^4, 10^3, and 500 Å), a pump (Varian 9010, Milan, Italy), a Prostar 355 RI detector (Varian Milan, Italy), and a software for computing M_w distribution (Galaxie Ws, ver. 1.8 Single-Instrument, Varian Milan, Italy) were used. The microsphere samples were dissolved in tetrahydrofuran (THF) under stirring at iced bath temperature. Sample solutions were filtered through a 0.45-μm filter (Millipore, Massachusset, USA) before injecting into the GPC system, and 50 μl were eluted with THF at 1 mL/min flow rate. The microspheres were analyzed before irradiation, immediately after irradiation and every 3 months along the 6-month stability conducted study. Polymers raw material were analyzed as control. Analyses were conducted in triplicate for each sample type.

The weight-average molecular weight (M_w) of each sample was calculated using monodisperse polystyrene standards

(M_w 1,000–500,000 Da). The data were processed as weight-average molecular weight (M_w), average molecular number (M_n), and polydispersity index (PI), and expressed as the percentages of the M_w, M_n, and IP variations with respect to the not irradiated polymer matrix obtained by Eqs. 1 and 2:

$$\%M_w\text{reduction} = \frac{M_{wnotirr} - M_{wirr}}{M_{wnotirr}} \times 100 \quad (1)$$

$$\%M_n\text{reduction} = \frac{M_{nnotirr} - M_{nirr}}{M_{nnotirr}} \times 100 \quad (2)$$

where $M_{w\ notirr.}$ and $M_{w\ irr.}$ are the weight-average molecular weights of not irradiated and irradiated microspheres, respectively; $M_{n\ notirr}$ and $M_{n\ irr.}$ are the average molecular numbers of not irradiated and irradiated microspheres, respectively.

IVM Determination by High Performance Liquid Chromatography

The HPLC analyses of IVM-loaded in the microspheres before and after irradiation were performed to evaluate the effects of irradiation treatment on the drug.

Samples preparation was performed as follows: 15 mg of IVM loaded microspheres were dissolved in 2 ml of methylene chloride, the solutions were kept overnight under a hood at room temperature conditions to promote the solvent evaporation and formation of a film containing polymer and IVM in the free form (not encapsulated). Fifteen milliliters of methanol were added to each sample to selectively extract IVM from polymer that is completely insoluble in methanol. After 2 h under magnetic stirring (300 rpm), the suspension was filtered through a 0.45-μm nylon membrane filter to separate the solid polymer from the IVM solution. 50 μl aliquots of IVM solution were analyzed by HPLC as follows. An Agilent 1260 HPLC apparatus (Agilent Technologies, Milan, Italy) consisting of a spectra system with a pump (1260 Infinity Quaternary Pump VL), UV detector (Agilent 1260 Series UV-visible detectors, multi-wavelength detector), and manual injector (Agilent 1260 Infinity Manual Injector) was used with a Zorbax Eclipse® Plus C18 Chromatography Column, 4.6×150 mm, 5 μm and a mobile phase made of a mixture of purified water (15 vol), methanol (34 vol), acetonitrile (51 vol), at flow rate of 1.5 ml/min and 254 nm detection wavelength.

IVM was determined from a standard calibration curve prepared starting from a stock solution containing 600 μg/ml IVM in 50 ml MeOH. Five milliliters of the stock solution was diluted into a 10-ml volumetric flask with methanol, to obtain a solution of 300 μg/ml of IVM in MeOH; 2.5, 1.25, and 0.625 ml of stock solution, respectively, were used to obtain the other calibration standards in the concentration of 150, 75, and 37.5 μg/ml. Each standard solution was analyzed in triplicate and each point of the calibration curve is the average of the three analyses. These standard solutions cover a hypothetical encapsulation efficiency ranging from 10 to 100%.

The corresponding placebo microparticles with and without TCP, respectively, were tested as controls. The encapsulation efficiencies were calculated according to the Eq. 3:

$$EE\% = \frac{\text{Actual drug content (mg)}}{\text{Theoretical drug content (mg)}} \times 100 \quad (3)$$

where the actual drug content corresponds to the IVM mg determined from the HPLC assay and the theoretical drug content is the IVM mg added to the polymer solution according to microparticle preparation protocol. Analyses were conducted in triplicate on each sample.

In Vitro Release Study

In vitro release test were carried out on microspheres before and after irradiation, and during the stability study. Float-a-Lyzer dialyzers (Float-a-Lyzer® G2, Spectra/Por®) equipped with 50 KDa M_w cutoff dialysis membrane were used as the test apparatuses. The dialysis membrane molecular weight cutoff was chosen on the basis of IVM molecular weight. A preliminary calibration test was carried out to exclude interactions between IVM and the membrane (data not reported). The following *in vitro* release test protocol was used. About 12 mg of IVM-loaded microspheres were transferred into the Float-a-Lyzer dialyzer and suspended in 5 mL of release medium (phosphate buffer supplemented with 0.2% hexadecyltrimethylammonium bromide (CTAB, pH 6.8) at 37°C. The dialyzer was then introduced into a glass cylinder containing 120-ml release medium, which was maintained in static conditions. Drug release was assessed by intermittently sampling the contents (1 ml) of the outer medium. The buffer was replaced immediately after sampling. The amount of released IVM was determined on each withdrawn sample by the same HPLC method used for IVM encapsulation efficiency determination (see above, IVM determination by high performance liquid chromatography).

Statistical Analysis

A minimum of five samples were used in all the experiments. The results are expressed as the mean value of at least five replicates±standard deviation (SD). Statistical analysis was carried out using a one-way analysis of variance with 95% confidence intervals. The error bars denote±sd ($n \geq 5$).

RESULTS AND DISCUSSION

Table II reports the results of particle size analysis performed on the microspheres before and after irradiation. The microspheres based on poly(DL-lactide)100DLE and on poly-(ε-caprolactone)100 CL19E, and added with TCP ($PL1_{TCP}$, $IVM1_{TCP}$, $PL4_{TCP}$, $IVM4_{TCP}$,) are always significantly bigger with respect to those without TCP, while the opposite behavior is highlighted for the poly-(ε-caprolactone) 100 CL10E and 100 CL20.4E-based microspheres added with TCP. Irradiation leads to significant increase of particle size in all the PL batches and to particle size decrease in all the PL_{TCP} ones. The effect of irradiation on particle size of the IVM-loaded batches

Table II. Results of Particle Size Analysis on the Not Irradiated and Irradiated Microspheres

	Particle size of not irradiated microsphere[a]				Particle size of irradiated microsphere[a]			
Batch #	d_{10} [μm]	d_{50} [μm]	d_{90} [μm]	SPAN	d_{10} [μm]	d_{50} [μm]	d_{90} [μm]	SPAN
PL1	64,415	145,028	301,318	1,633	77,943	162,01	330,538	1,559
$PL1_{TCP}$	78,532	180,852	409,318	1,829	81,472	178,213	379,551	1,673
IVM1	59,372	152,043	314,925	1,681	69,52	175,183	350,22	1,602
$IVM1_{TCP}$	53,789	194,699	389,052	1,722	63,713	186,761	373,942	1,661
PL2	36,29	81,689	533,463	6,086	39,207	128,767	620,186	4,512
$PL2_{TCP}$	33,815	75,789	489,819	2,058	35,243	83,961	220,033	2,201
IVM2	41,445	100,169	211,407	1,697	41,381	97,21	233,018	1,766
IVM2 $_{TCP}$	53,371	137,576	281,762	1,66	49,962	126,319	261,161	1,672
PL3	109,11	248,373	459,425	1,41	70,429	275,359	838,118	2,788
$PL3_{TCP}$	52,866	217,67	496,085	2,036	58,141	231,59	439,678	1,863
IVM3	167,022	*242,326*	*372,476*	1,276	50.036	125.72	260.3	1.672
$IVM3_{TCP}$	41,049	103,164	274,214	2,26	30,68	113,193	306,841	2,44
PL4	88,979	207,677	448,85	1,733	72,389	216,017	505,639	2,006
$PL4_{TCP}$	95,936	252,548	598,413	1,99	102,631	203,433	420,477	1,562
IVM4	103,481	260,597	235,83	1,659	95,992	287,01	645,372	1,914
$IVM4_{TCP}$	101,303	272,042	280,662	1,762	117,151	270,145	558,222	1,633

[a] sd<5%

is not consistent and apparently is not dependent on their composition since IVM1, IVM2, IVM4, $IVM3_{TCP}$, and $IVM4_{TCP}$ particle size increases upon irradiation. The results of particle size analysis are confirmed by SEM analysis. Electronic microscopy permits to highlight the regular shape of the microsphere and the absence of fibers or irregular particles. IVM1 and $IVM1_{TCP}$ microparticles show some crystals on their surface. It can be hypothesized that they are IVM crystals because of the very low drug aqueous solubility, and it can be ascribed to fast solvent evaporation. The higher solvent evaporation temperature used for the preparation of poly(DL-lactide)100DLE microparticles (IVM1, $IVM1_{TCP}$), with respect to the poly-(ε-caprolactone) ones, increases solvent evaporation rate and improves drug migration toward microsphere surface; the low drug aqueous solubility causes drug precipitation at the organic solvent aqueous phase interface. Due to the different polymer physical-chemical characteristics and to the different process conditions used, poly-(ε-caprolactone) microsphere surface morphology is more smooth, as shown in the examples in Fig. 1. A comparison, by SEM analysis, between the microsphere before and after irradiation, shows that irradiation did not cause either their rupture, collapse, or surface damage. The result, partially in disagreement with those previously discussed in the literature by the authors for polylactide-co-glycolide-based microparticles (17,18), can be explained by the high hydrophobicity both of poly-(ε-caprolactone) and poly (DL-lactide).

The results of GPC analyses are reported in Table III as average M_w and M_n reduction percentages, calculated immediately after irradiation with respect to the not irradiated polymer raw material and microspheres polymer matrix, respectively. They should be differentiated because irradiation can play a different effect on the material depending also on its physical structure (microsphere or powder). Gamma irradiation induced M_w and M_n reduction both of the polymer raw materials and the microspheres polymer matrix. Polymer chemical compositions and their starting molecular weights significantly affect the entity of gamma irradiation effects. The greatest $_w$ *reduction is highlighted in polylactide 100 DL 7E (50.1, 60.5, 58.3%), both as raw material and as microsphere polymer matrix. Lower molecular weight reduction is shown in the poly-(ε-caprolactone) raw materials and the related microsphere polymers matrices, 100 CL10E, 100 CL20.4E e 100 CL19E (21.9, 45.0, and 46%; 17.5, 23.6, and 33.4%; and 15, 16.8, and 15.3%). Moreover, it is possible to state that gamma irradiation damages, in terms of* M_w *and* M_n *reduction, are higher for lower* M_{ws} *polymers (100 DL 7E and 100 Cl 10E).*

Upon irradiation, the microsphere polymer matrices of all the placebo microspheres and TCP-loaded placebo microspheres show significant higher reduction in polymer M_w with respect to the corresponding polymer raw material (Table III). The phenomenon can be ascribed to the microsphere microstructure (*i.e.*, porosity and particle size) that, increasing the microsphere surface area with respect to the polymer in bulk powder, leads to increase the interactions of the polymer microsphere matrix with gamma irradiation thus rendering the microspheres polymer matrix more sensitive than the corresponding polymer raw material. Since TCP was added to the microspheres as radical scavenger, it was expected to exert stabilization both on the polymer and the drug, but surprisingly TCP addition to placebo microspheres did not affect molecular weight reduction upon irradiation.

Nevertheless, for all the tested polymers, M_n was more sensitive toward sterilization treatment than M_{ws}.

Several authors describe higher degradation for high molecular weight polymers, after irradiation (15,19–21) and on the consolidate scientific literature basis, cleavage is known to be the gamma irradiation-induced polymer degradation mechanism. Kissel *et al.* (19) hypothesized a random chain scission and an unzipping mechanism as reported in the scheme in Fig. 2. The last hypothesized mechanism involves mainly the terminal groups of polymer chains and, as demonstrated by

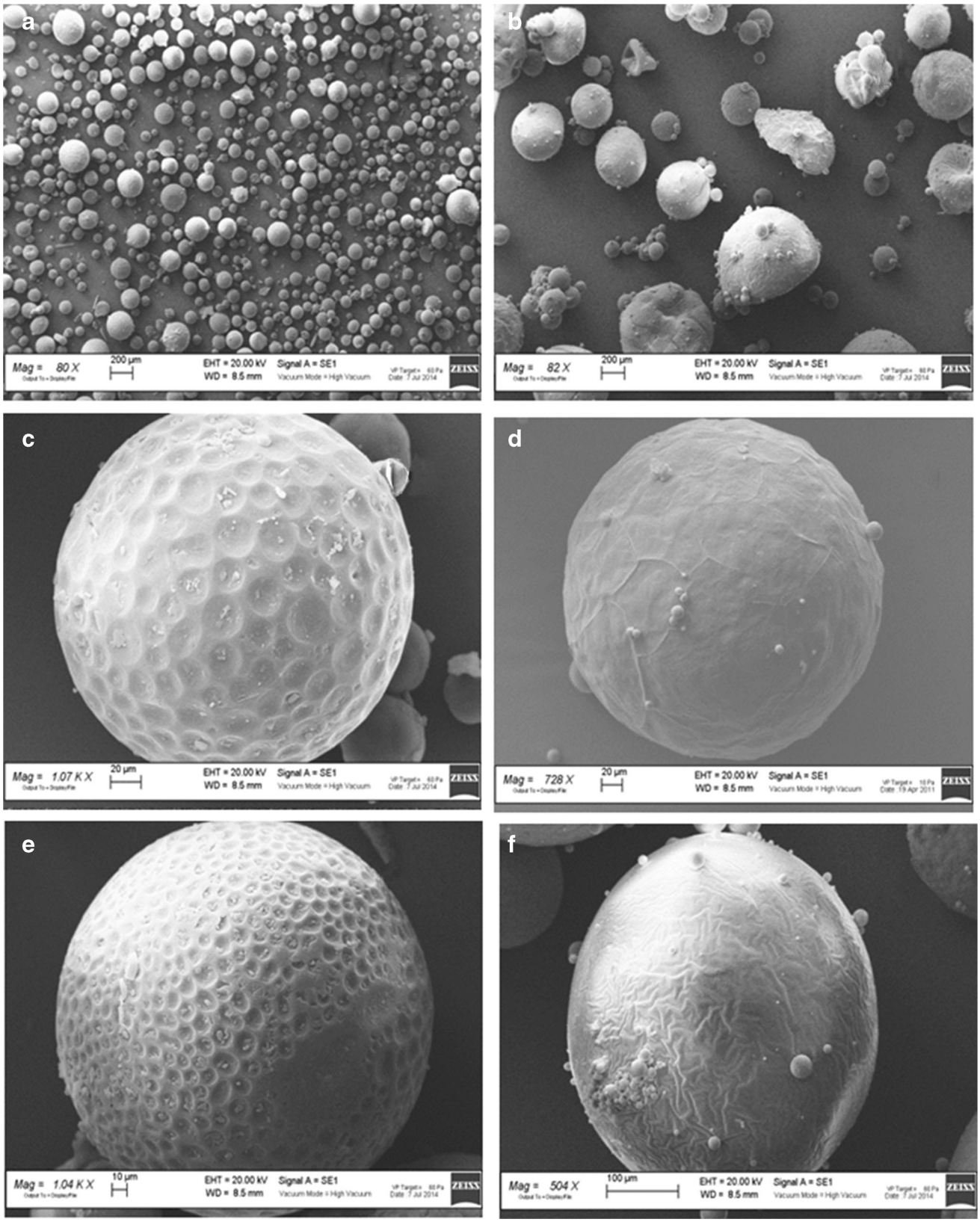

Fig. 1. SEM images of **a** $IVM1_{TCP}$ at ×80 and **c** $IVM1_{TCP}$ at 1.04 kX magnifications before irradiation; **b** $IVM4_{TCP}$ at ×82 and $IVM4_{TCP}$ at ×728 magnifications before irradiation; **e** $IVM1_{TCP}$ at 1.04 kX magnification after irradiation; **f** $IVM4_{TCP}$ at ×524 magnification after irradiation

the authors, it is responsible of an increase in monomers and oligomers upon irradiation, causing faster reduction of average Mn with respect to M_w. Moreover, polymer chain flexibility increases as M_w decreases, and in these conditions,

Table III. Effects of Gamma Irradiation on Polymers and Placebo Microspheres M_w and M_n

Polymer	Microsphere batch	ΔM_w[a,b] (%)	ΔM_n[a,c] (%)	G_s/G_x	G_s/G_x
100 DL 7 E	Polymer raw material	50.1	61.9	36.52	36.52
	PL1	60.5	64.0	33.50	13.52
	$PL1_{TCP}$	58.3	74.0	20.45	10.64
	IVM1	50.0	62.0	33.21	11.77
	$IVM1_{TCP}$	50.8	74.0	21.33	6.67
100 CL 10 E	Polymer raw material	21.9	54.8	5.93	5.93
	PL2	45.0	62.4	10.66	11.23
	$PL2_{TCP}$	46.0	69.0	11.31	9.06
	IVM2	32.6	60.0	10.51	5.38
	$IVM2_{TCP}$	24.0	46.0	11.28	4.14
100 CL 20.4 E	Polymer raw material	17.5	35.9	6.24	6.24
	PL3	23.6	38.0	8.52	5.20
	$PL3_{TCP}$	33.4	41.0	8.43	4.83
	IVM3	17.0	32.0	8.44	7.09
	$IVM3_{TCP}$	20.7	59.0	8.34	5.31
100 CL 19 E	Polymer raw material	15.0	17.2	15.42	15.42
	PL4	16.8	28	21.67	7.97
	$PL4_{TCP}$	15.3	33.4	20.78	4.49
	IVM4	12.3	21.5	21.05	12.46
	$IVM4_{TCP}$	9.97	28.0	20.88	4.83

[a] sd<5%
[b] ΔM_w molecular weight reduction percentage calculated according to Eq. 1
[c] ΔM_n molecular number reduction percentage calculated according to Eq. 2

the primary free radicals generated by irradiation can recombine with other radicals starting other reactions according to their decay reaction. The entity of scission and crosslinking reactions can be determined on the irradiated samples computing the yields of chemical scission (G_s) and crosslinking (G_x) reactions according to the following equations on the base of GPC average M_w and M_n data:

$$\frac{1}{M_{wirr}} = \frac{1}{M_{wnonirr}} + \frac{(G_s - 4G_x)}{2} D \times 1.038 \times 10^{-6} \quad (4)$$

$$\frac{1}{M_{nirr}} = \frac{1}{M_{nnonirr}} + (G_s - G_x) D \times 1.038 \times 10^{-6} \quad (5)$$

The yields of chemical scission (G_s) and crosslinking (G_x) reactions are defined as the number of reactions each 100 electronvolts absorbed energy, $M_{wnonirr}$ and $M_{nnonirr}$ are the average molecular weights and molecular numbers of the irradiated and not irradiated samples, respectively, and D is the irradiation dose (25 kGy).

As stated in the literature (5), G_s/G_x ratio greater than 4, indicates that the polymer chain scission mechanism prevails on the crosslinking one.

On the basis of the GPC data, G_s and G_x have been here calculated and reported in Table III, the G_s/G_x ratios>4 demonstrate that chain scission reactions always prevail both on the polymers and placebo microspheres, independently on the presence of TCP.

No further variations in M_w, M_n, and PI were detected in the 6-month the stability study (data not reported).

IVM loading into the microspheres (batches IVM 1–4) always leads to statistically significant reduction of the polymer matrix degradation with respect to placebo batches (PL1-4), as reported in Table III. The contemporary presence of IVM and TCP in the polymer matrix ($IVM1_{TCP}$—$IVM4_{TCP}$) leads to further polymer stabilization toward irradiation only in batches $IVM2_{TCP}$ and $IVM4_{TCP}$. The results are consistent

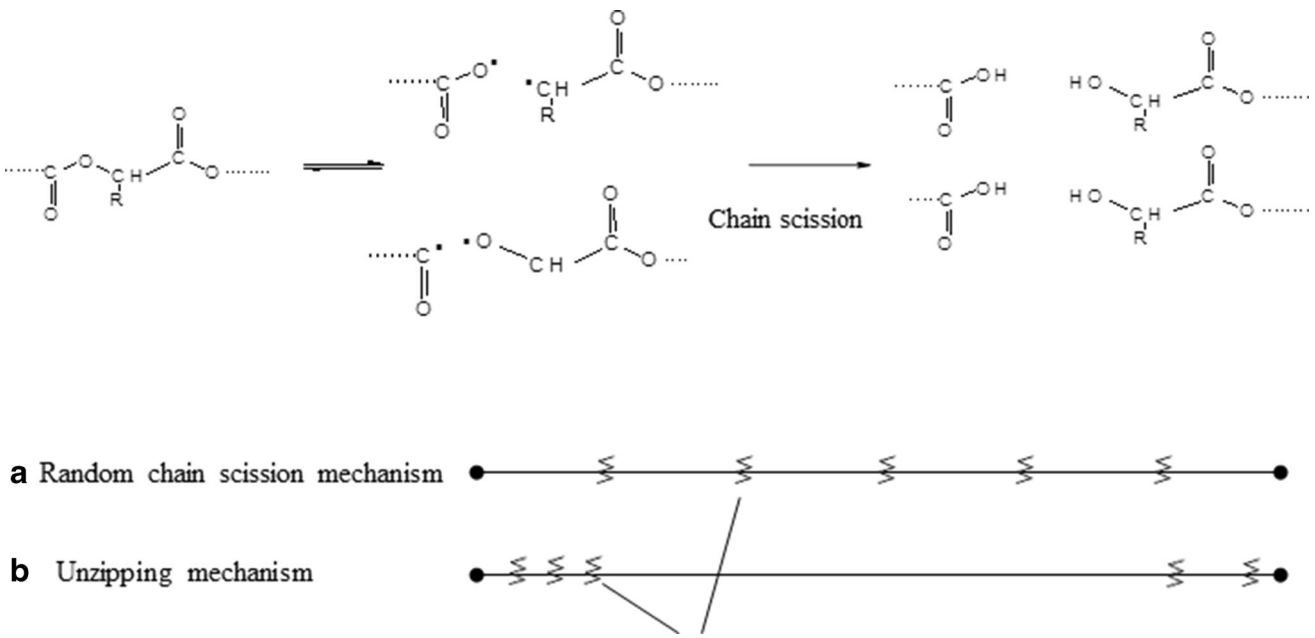

Fig. 2. Schematic representation of the suggested polymer degradation mechanism: **a** random chain scission and **b** unzipping

with IVM properties: the drug is highly sensitive to irradiation and when in intimate contact with the polymers, it acts as radical scavenger for the polymers, whose sensitivity to irradiation is lower. For the same reason, TCP alone does not seem to stabilize the polymers toward irradiation, while it stabilizes the drug, as demonstrated by the results of HPLC analysis (Fig. 3, Table IV). The presence of both IVM and TCP in the microsphere polymer matrix has a synergistic effect. It was hypothesized that increasing the amount of TCP loaded in the microspheres could improve the TCP antioxidant effect. However, the preliminary studies conducted showed that the highest TCP percentage loaded in the microspheres (0.85%) is achieved by 1% TCP addition to the polymer solution. Addition of higher amounts of TCP did not lead to increase TCP loading since higher amounts are lost along the microsphere preparation process (data not reported).

The effects induced on IVM by irradiation were determined by HPLC analysis of IVM extracted from the microspheres before and after irradiation. The chromatograms in Fig. 3 show two peaks for IVM standard solution before irradiation (red line Fig. 3a), a main peak at 16.4 min and a smaller one at 12.2 min, that keep unchanged in the microspheres as shown, as an example, in the chromatograms of IVM extracted from the IVM1 and IVM1$_{TCP}$ microspheres batches (Fig. 3b and c, red lines), demonstrating that the microsphere preparation process does not have detrimental effect on the drug.

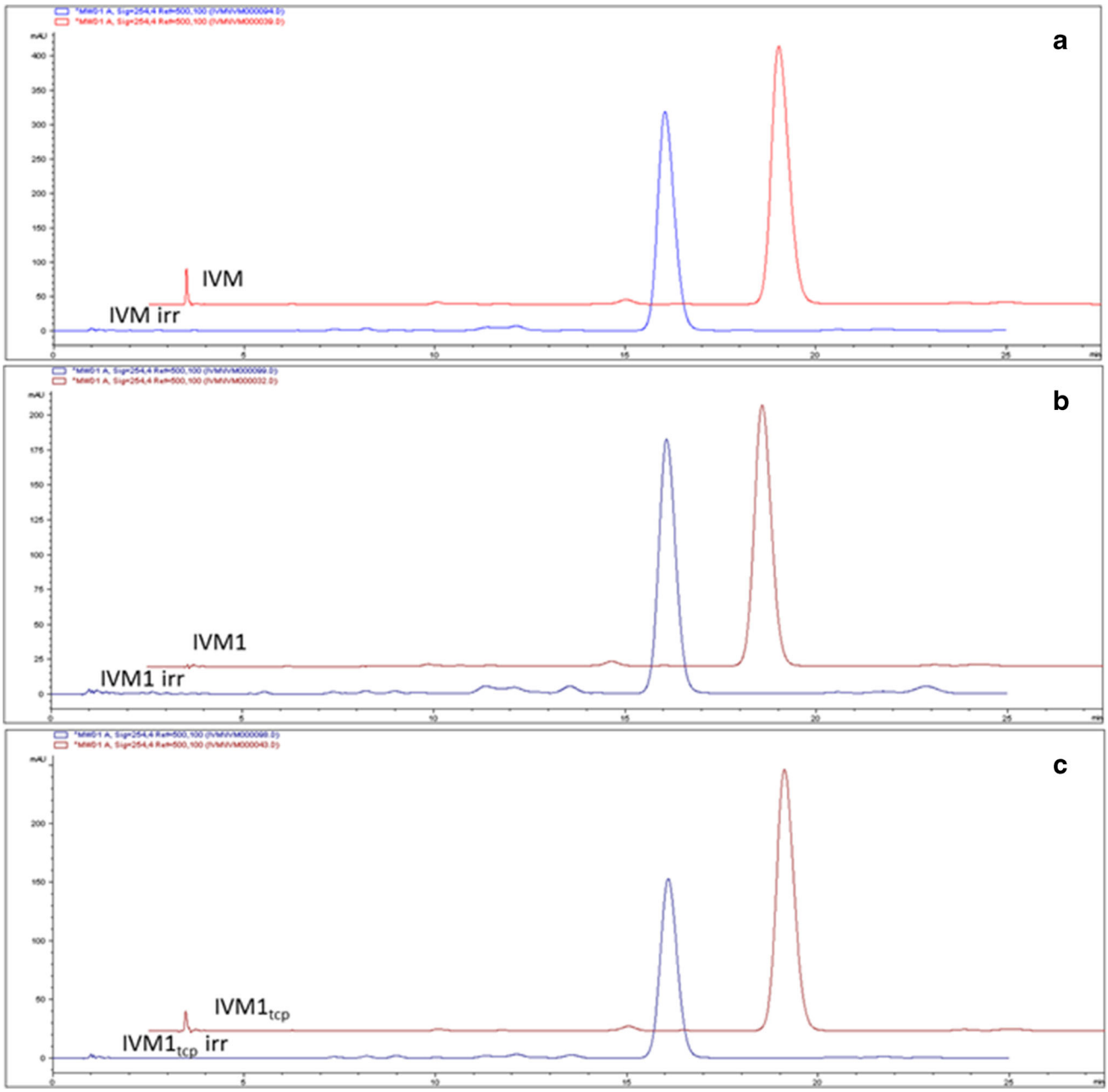

Fig. 3. HPLC chromatograms of IVM from standard solution (IVM), and from batches IVM1 and IVM1$_{TCP}$: *red line* before irradiation and in *blue line* after irradiation

Table IV. Stability Study on the Not Irradiated and Irradiated IVM- and IVM_{TCP}-loaded Microspheres: IVM Encapsulation Efficiency

Batch #	Not irradiated microspheres EE %[a]			Irradiated microspheres EE %[a]		
	t_0	$t_{3\ months}$	$t_{6\ months}$	t_0	$t_{3\ months}$	$t_{6\ months}$
IVM1	71.65	60.30	47.29	54.79	43.62	30,16
$IVM1_{TCP}$	76.50	76.00	76.20	63.59	59.91	57.59
IVM2	84.91	72.21	56.04	62.84	50.71	35.60
$IVM2_{TCP}$	79.80	79.01	79.60	71.92	68.65	64.30
IVM3	81.31	68.70	53.67	62.89	51.00	33.81-
$IVM3_{TCP}$	81.40	81.30	80.9	74.07	71.12	66.09
IVM4	64.74	54.33	42.01	46.26	38.25	26.10-
$IVM4_{TCP}$	67.37	67.26	67.20	59.44	56.94	53.22

[a] sd<5%

Further peaks, at 5–15 min and 20–25 min, always appear in the HPLC chromatograms upon irradiation, as highlighted in Fig. 3 (blue lines) for IVM extracted from irradiated IVM1 and $IVM1_{TCP}$, and in Fig. 4a, b reporting the chromatograms magnifications of IVM extracted from IVM1, $IVM1_{TCP}$, IVM4, and $IVM4_{TCP}$. The same peaks, generated upon irradiation, are highlighted also in the samples of irradiated IVM raw material (Fig. 3a, IVM, blue line); therefore, they can be attributed to IVM degradation products. Moreover, the area under the peaks produced by irradiation, is lower in the TCP loaded batches demonstrating a possible radical scavenger effect of TCP toward IVM. The overall results demonstrate the efficacy of TCP as radical scavenger toward IVM.

The results of microspheres stability evaluation performed on the not irradiated and irradiated microspheres stored at 4°C are reported in Table IV as IVM encapsulation

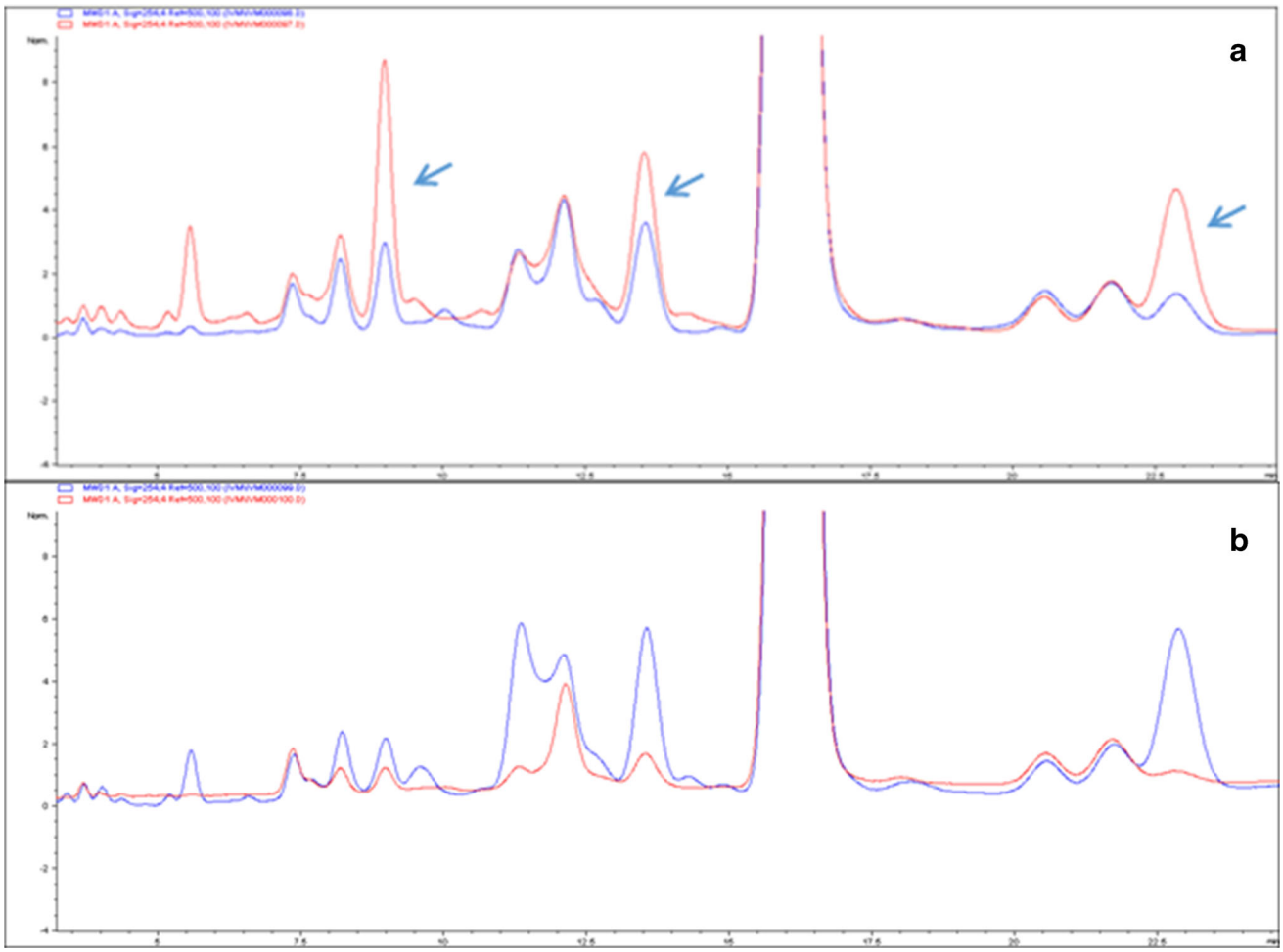

Fig. 4. Magnification of HPLC chromatograms of irradiated IVM from: **a** batch IVM1 and **b** batch IVM4, before irradiation (*red line*) and after irradiation (*blue lines*)

Table V. Stability Study on the Not Irradiated and Irradiated IVM- and IVM_{TCP}-Loaded Microspheres: IVM Release from Irradiated and not Irradiated Microspheres

	IVM release % from irradiated microspheres tested immediately after preparation (time 0)			IVM release % from not irradiated microspheres tested immediately after preparation and irradiation (time 0)				IVM release % from irradiated microspheres tested after 6-month storage (time 6)				IVM release % from not irradiated microspheres tested 6-month storage after irradiation (time 6)			
Batch #	24 h	1 month	3 months	6 months	24 h	1 month	3 months	6 months	24 h	1 month	3 months	6 months	24 h	1 month	3 months	6 months
$IVM1_{TCP}$	15.1±0.2	20.0±0.2	20.3±0.4	90.5±0.1	18.6±0.1	23.4±0.3	25.3±0.2	100±0.2	15.3±0.1	21.0±0.2	21.8±0.3	94.0±0.4	19.6±0.1	24.2±0.3	26.8±0.2	100±0.3
$IVM2_{TCP}$	37.5±0.1	78.2±0.4	80.3±0.3	100±0.3	41.2±0.2	85.0±0.1	92.3±0.4	100±0.2	38.2±0.1	78.0±0.2	82.3±0.2	100±0.3	43.0±0.2	88.6±0.1	96.1±0.4	100±0.2
$IVM3_{TCP}$	25.0±0.2	84.6±0.1	100±0.3	–	27.0±0.3	91.5±0.4	100±0.4	–	25.8±0.2	85.0±0.2	100±0.2	–	28.2±0.3	94.7±0.2	100±0.3	–
$IVM4_{TCP}$	20.3±0.3	50.5±0.3	81.0±0.2	100±0.4	22.4±0.3	55.9±0.3	90.5±0.4	100±0.2	21.3±0.1	52.3±0.2	82.0±0.1	100±0.2	22.9±0.3	57.3±0.4	94.2±0.3	100±0.2

efficiency, and in Table V as % of IVM *in vitro* released from the microspheres. These results involve the influence of both the polymer and TCP on the drug and polymer microsphere matrix stability.

IVM encapsulation efficiency is in the 67.37 and 84.91% range, and it is not significantly affected by TCP addition, with the exception of the polylactide-based $IVM1_{TCP}$ batch whose encapsulation efficiency increases of about 9% with respect to IVM1 batch. TCP addition (batches $IVM1_{TCP}$–$IVM4_{TCP}$) stabilizes IVM in the 6-month storage time evaluated.

Irradiation leads to a significant encapsulation efficiency reduction in IVM1–IVM4 batches of about 23–27%. TCP addition ($IVM1_{TCP}$–$IVM4_{TCP}$) protects IVM during irradiation as highlighted by the results reported in Table IV showing that encapsulation efficiency reduction ranges between 8.6 and 1.7% in the irradiated batches containing TCP ($IVM1_{TCP}$–$IVM4_{TCP}$) immediately after irradiation (time 0). The encapsulation efficiency in the irradiated $IVM1_{TCP}$–$IVM4_{TCP}$, during the 6-month stability study, decreases between 5 and 6%, and it is about 30% in the IVM1–IVM4 batches, thus highlighting that TCP stabilizing effect lasts in the 6-month storage after irradiation.

The results of *in vitro* release tests performed on the not irradiated and irradiated microspheres, immediately after preparation and sterilization (time 0) and after 6-month storage (time 6), are reported in Table V as percentage of released IVM. Since the stabilizing effect of TCP was considered unavoidable in a commercial formulation, only the data of IVM-loaded microspheres containing TCP are reported. The results show that IVM release is significantly slower from polylactide microparticles ($IVM1_{TCP}$) with respect to the poly-(ε-caprolactone) ones ($IVM2_{TCP}$–$IVM4_{TCP}$): this is consistent with the low Tg of poly-(ε-caprolactone) polymers, and the consequent rubbery state of the microparticles at 37°C (simulated physiologic conditions). $IVM3_{TCP}$ batch shows the fastest IVM release rate, that is completed (100%) in 3 months. The behavior can be due to the small particle size of this batch (see Table II). Drug release rate always significantly decreases upon irradiation between 9 and 10% for the poly-(ε-caprolactone) microparticles (batches ($IVM2_{TCP}$–$IVM4_{TCP}$), and even more (18%) for the polylactide-based microparticles (batch $IVM1_{TCP}$). Drug release from the microspheres is unchanged after 6-month storage, since the variations are always within 5%.

CONCLUSIONS

The achieved results confirmed the IVM instability issue, in such a way that, even when entrapped in a polymer matrix, it should be stabilized with an antioxidant (TCP) to obtain a stable pharmaceutical product. Irradiation always leads both to the drug and the polymer degradation, however, TCP addition can constrain the phenomenon within a 8–10% polymer degradation when combined to poly-(ε-caprolactone) and has a synergistic stabilizing effect on the drug.

The microsphere characteristics in terms of their structure and drug release behavior depend on the polymer matrix and could be modulated in by mixing microspheres of different composition in the same product.

The overall conclusion is that the formulation, and terminal sterilization by gamma irradiation, of a microparticulate IVM-loaded delivery system for veterinary use is feasible.

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