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Production of GMP-grade radioactive holmium loaded poly(L-lactic acid) microspheres for clinical application

S.W. Zielhuis [a,*], J.F.W. Nijsen [a], R. de Roos [a], G.C. Krijger [b], P.P. van Rijk [a], W.E. Hennink [c], A.D. van het Schip [a]

[a] *Department of Nuclear Medicine, University Medical Center, Utrecht, The Netherlands*
[b] *Department of Radiation, Radionuclides and Reactor, Delft University of Technology, The Netherlands*
[c] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands*

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Abstract

Radioactive holmium-166 loaded poly(L-lactic acid) microspheres are promising systems for the treatment of liver malignancies. The microspheres are loaded with holmium acetylacetonate (HoAcAc) and prepared by a solvent evaporation method. After preparation, the microspheres (Ho-PLLA-MS) are activated by neutron irradiation in a nuclear reactor. In this paper, the aspects of the production of a (relatively) large-scale GMP batch (4 g, suitable for treatment of 5–10 patients) of Ho-PLLA-MS are described.

The critical steps of the Ho-PLLA-MS production process (sieving procedure, temperature control during evaporation and raw materials) were considered and the pharmaceutical quality of the microspheres was evaluated.

The pharmaceutical characteristics (residual solvents, possible bacterial contaminations and endotoxins) of the produced Ho-PLLA-MS batches were in compliance with the requirements of the European Pharmacopoeia. Moreover, neutron irradiated Ho-PLLA-MS retained their morphological integrity and the holmium remained stably associated with the microspheres; it was observed that after 270 h (10 times the half-life of Ho-166) only $0.3 \pm 0.1\%$ of the loading was released from the microspheres in an aqueous solution.

In conclusion, Ho-PLLA-MS which are produced as described in this paper, can be clinically applied, with respect to their pharmaceutical quality.

Keywords: Holmium; Microspheres; PLLA; GMP; CFU; Endotoxins; Stability

1. Introduction

1.1. Holmium-166 loaded microspheres

Radionuclide loaded microspheres are promising systems for the treatment of liver malignancies. It has been shown that microspheres with a size of 20–50 μm, when administered into the hepatic artery of patients suffering from liver malignancies, lodge in and around the tumour, and irradiate the surrounding tissue (Nijsen et al., 2002b). In view of its physical properties, holmium-166 is the ideal radionuclide for such therapies. Namely, holmium is the only element that can be both neutron-activated to a beta- and gamma-emitter with a logistically favourable half-life and visualized by MRI (Nijsen et al., 2002b, 2004; Zielhuis et al., 2005b). Using a solvent evaporation technique, non-radioactive holmium-165 as its acetylacetonate complex (HoAcAc) can be incorporated into poly(L-lactic acid) (PLLA) microspheres. Subsequently, the microspheres (Ho-PLLA-MS) can be made radioactive by neutron irradiation (Nijsen et al., 1999).

Before Ho-PLLA-MS can be clinically applied in a phase-1 trial, the entire production procedure must comply with the Good Manufacturing Practice regulations (GMP) promulgated by the European Agency for the Evaluation of Medicinal Products (EMEA) (De Vos et al., 2005). Although many papers have been published on the production of microspheres, none of the papers describe the setting up of a GMP production process. Furthermore, for clinical application in human patients, production of microspheres at milligram-scale is insufficient; for

* Corresponding author at: Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Tel.: +31 30 2506295; fax: +31 30 2542531.
E-mail address: S.Zielhuis@azu.nl (S.W. Zielhuis).

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the treatment of one patient with Ho-PLLA-MS, a quantity of 500–1000 mg is needed (Nijsen et al., 1999). In this paper the GMP-production of a relatively large batch (4 g) of Ho-loaded PLLA microspheres is described in detail. The critical steps of the Ho-PLLA-MS production process were identified and investigated, and the pharmaceutical quality of the microspheres before and after neutron irradiation was evaluated.

1.2. Requirements for clinical application

The effects of neutron irradiation on the characteristics of Ho-PLLA-MS have been extensively studied in previous papers (Nijsen et al., 1999, 2002a; Zielhuis et al., 2005a). Although the molecular weight of the PLLA decreases due to the irradiation, the chemical entity of the polymer is retained (Nijsen et al., 2002a). However, the consequences of this decrease in molecular weight, in terms of safety for the patient, need to be studied. It is also important for therapeutic radiopharmaceuticals to have a high radiochemical stability. The release of radioactive holmium from the microspheres in the human body, which might lead to distribution of radioactivity to organs other than the liver, may lead to serious adverse events and must therefore be prevented. Additionally, the microspheres must retain their size and shape after irradiation, in order to ensure a selective biodistribution and to prevent excessive exposure of non-target organs to the irradiation.

In order to be used for clinical application, radioactive Ho-PLLA-MS must also fulfill the following requirements of the European Pharmacopoeia (Ph. Eur., 2002):

- The absence of microbial contamination.
- A limited amount of endotoxins. The limit for parenteral drugs is 5.0 EU/kg body weight.
- An acceptable amount of residual solvents (which are used during the solvent evaporation process). The ICH-guidelines (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) give limits of 60 and 600 ppm for chloroform and dichloromethane, respectively (B'Hymer, 2003).

Once Ho-PLLA-MS are produced according to GMP and meet the above criteria, clinical application in cancer treatment can be initiated.

2. Materials and methods

2.1. Materials

All chemicals were commercially available and used as obtained. Acetylacetone, 2,4-pentanedione (AcAc; >99%), chloroform ($CHCl_3$; HPLC-grade), poly(vinyl alcohol) (PVA; molecular weight 30,000–70,000, 88% hydrolyzed), ammonium hydroxide (NH_4OH; 29.3% in water) and holmium(III) acetylacetonate hydrate (HoAcAc; 99.9%) were supplied by Sigma–Aldrich (Steinheim, Germany). Sodium hydroxide (NaOH; 99.9%) was purchased from Riedel-de Haën (Seelze, Germany). Holmium(III) chloride hexahydrate ($HoCl_3{\cdot}6H_2O$; 99.9%) and dichloromethane (CH_2Cl_2; HPLC-grade) were obtained from Phase Separations BV (Waddinxveen, The Netherlands). Poly(L-lactic acid) (PLLA; inherent viscosity 1.14 dl/g in chloroform 25 °C) was purchased from Purac Biochem (Gorinchem, The Netherlands). Hydrochloric acid (HCl; 37%), sodium azide (NaN_3; 99%), disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$; 99%) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$; 99%) were purchased from Merck (Darmstadt, Germany). Water for injections (in accordance with the European Pharmacopoeia (Ph. Eur., 2002)) was obtained from Braun (Melsungen, Germany).

2.2. Preparation of HoAcAc and microspheres

The equipment used for preparation of Ho-PLLA-MS by solvent evaporation and the subsequent sieving and drying steps, was made of borosilicate glass, stainless steel, silicon rubber or polytetrafluoroethylene, and was steam sterilised at 121 °C for 30 min at the Central Sterilisation Department of the University Medical Center Utrecht. Preparation of HoAcAc and Ho-PLLA-MS was carried out in a class-D clean room (Ph. Eur., 2002).

HoAcAc was prepared as described previously (Nijsen et al., 1999). Acetylacetone (180 g) was dissolved in water (1080 g) and the pH of this solution was brought to 8.50 with an aqueous solution of ammonium hydroxide. Holmium chloride (10 g dissolved in 30 ml water) was immediately added to this solution. After 15 h incubation at room temperature, the formed HoAcAc crystals were collected by centrifugation and washed with water.

HoAcAc (10 g) and PLLA (6 g) were dissolved in chloroform (186 g) (solution A). Twenty grams of PVA was dissolved in 1000 g of boiling water, and the solution was allowed to cool to room temperature (solution B). Solution A was immediately added to solution B and the mixture was stirred (500 rpm) for 40 h and the temperature during the solvent evaporation process was maintained at room temperature using a setup as shown in Fig. 1. In order to accelerate evaporation of the solvent, nitrogen gas (filtered through a 0.22 μm HEPA-filter) was flushed (15 l/min) over the stirring mixture (Fig. 1). The formed microspheres were collected by centrifugation and washed three times with water, three times with 0.1 M HCl and finally three times with water. The washed microspheres were fractionated according to size using stainless steel sieves with a pore size of 20 and 50 μm, and by using 1 l of water (Fig. 2, method A). The sieving system consisted of an Electromagnetic Sieve Vibrator (EMS 755) combined with an Ultrasonic Processor (UDS 751) (both from Topas GmbH, Dresden, Germany).

The collected microspheres (∼4 g) were dried at 70 °C under vacuum for 5 h using a rotating glass oven (B-580 GKR, Buchi, Zurich, Switzerland). After drying, the microspheres (500 mg) were packed in high-density polyethylene vials (type A, Posthumus Products, Beverwijk, The Netherlands).

In addition to the standard preparation method as described above, microspheres were also prepared by varying the following four parameters:

1. The sieving procedure. The obtained microspheres were suspended in water (200 ml) and fractionated according to size

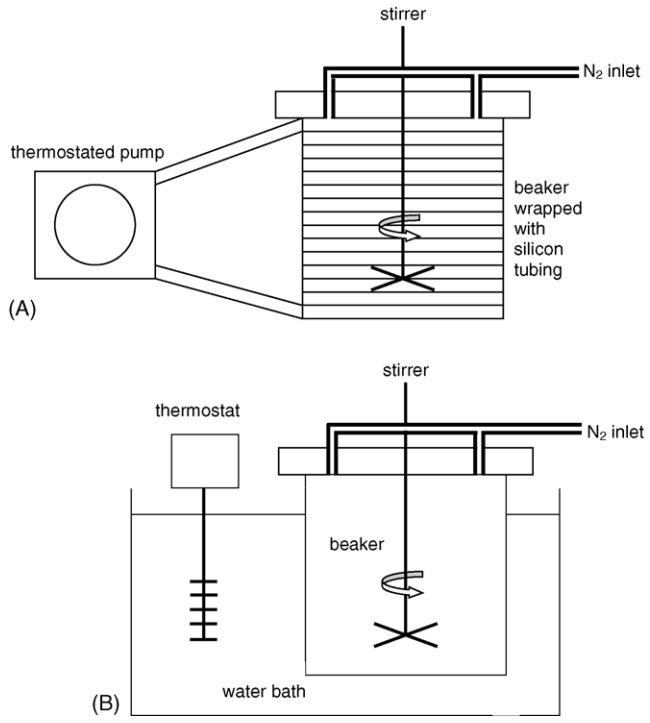

Fig. 1. Temperature control during the solvent evaporation process. Set up A: a glass beaker (inner diameter 13 cm, height 16 cm, volume 1.5 l) with four 1 cm (depth) baffles was wrapped with a silicon tube (external diameter 12 mm). The silicon tube was connected to a thermostated chamber equipped with a circulator (Thermo Haake DC 10, Karlsruhe, Germany). The temperature of the chamber was maintained at 37 °C during stirring with a four-bladed propeller stirrer (type R 1345, IKA® Werke GmbH and Co., KG, Staufen, Germany). Set up B: the same beaker was placed in a water bath of which the temperature was maintained at 25 °C.

using stainless steel sieves of 20 and 50 μm with a sprinkler system (Analysette 3 system, Fritsch GmbH, Idar Oberstein, Germany) using 8 l of water (Fig. 2 method B).

2. The organic solvent. Dichloromethane (DCM) instead of chloroform was used as a solvent in the preparation of Ho-PLLA-MS since higher residues of DCM are allowed by the ICH-guidelines: the ICH-guidelines give limits of 60 and 600 ppm for chloroform and dichloromethane, respectively (B'Hymer, 2003).
3. The origin of HoAcAc. Commercially available HoAcAc obtained from Sigma–Aldrich (Steinheim, Germany) instead of HoAcAc as synthesised by ourselves.
4. The temperature control during the solvent evaporation process (Fig. 1). For the preparation of Ho-PLLA-MS on a gram-scale with a solvent evaporation process, large amounts of chloroform (186 g) need to be evaporated. This will probably result in a (drastic) lowering of temperature of the water/chloroform mixture and, therefore requires controlled maintenance of temperature in order to ensure evaporation of the organic solvent. The temperature of the PVA solution during solvent evaporation was, therefore monitored and maintained at room temperature.

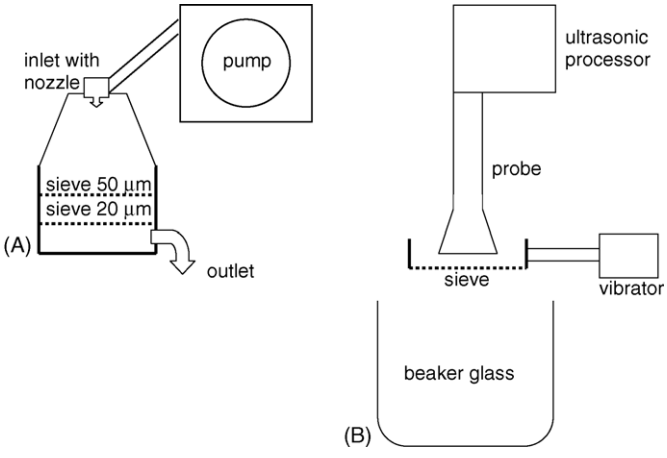

Fig. 2. Sieving procedure of microspheres. (A) Standard sieving procedure. (B) Sieving procedure using an ultrasonic probe and vibrator.

2.3. Neutron irradiation

Ho-PLLA-MS (~250 mg) packed in polyethylene vials were neutron irradiated at the Reactor Institute in Delft, The Netherlands with a thermal neutron flux of 5×10^{12} cm^{-2} s^{-1} for 6 h. Analyses of the neutron-irradiated microspheres were performed, after decay for 1 month in closed vials, having been stored at room temperature.

2.4. Determination of holmium content in microspheres

The holmium content in microspheres was determined by a complexometric titration as described previously (Zielhuis et al., 2005a).

2.5. Particle size distribution and scanning electron microscopy (SEM)

The particle size distribution of radiated and non-radiated microspheres was determined using a Coulter Counter (Multisizer 3, Beckman Coulter, The Netherlands) equipped with a 100-μm orifice.

The surface morphology of the Ho-PLLA-MS was investigated by SEM using a Philips XL30 FEGSEM. A voltage of 5 or 10 kV was applied. Samples of microspheres were mounted on aluminium stubs and sputter-coated with a Pt/Pd layer of about 10 nm.

2.6. Microbiological examination

Microbiological examination of three batches of Ho-PLLA-MS before neutron irradiation was carried out using the European Pharmacopoeia method 2.6.12, 2.6.13 and 2.6.14 (Ph. Eur., 2002) and were performed by Bactimm B.V., Nijmegen, The Netherlands. The batches were prepared according to the standard preparation method as described in Section 2.2. The temperature of the water/chloroform mixture during the solvent evaporation process of these batches was maintained at room temperature, by method A as represented in Fig. 1.

2.7. Residual solvents

The residual solvent levels of three batches of Ho-PLLA-MS after neutron irradiation were determined using European Pharmacopoeia method 2.4.24, 'identification and control of residual solvents for water insoluble substances' (Ph. Eur., 2002), and were carried out by Farmalyse B.V., Zaandam, The Netherlands.

2.8. Release of holmium

The release of holmium from neutron irradiated Ho-PLLA-MS (after decaying for 1 month, see Section 2.3) was tested in triplicate, with a method in analogy with that described by Hafeli et al. (2001) which is comparable to the rotating basket method as described in the European Pharmacopoeia (method 2.6.12) (Ph. Eur., 2002). Microsphere samples (250 mg) were dispersed in 50 ml of an isotonic phosphate buffer (174 mmol, pH 7.4) in a 50 ml test tube (Greiner-Bio One, Frickenhausen, Germany). The tubes (in triplicate) were closed with a cap containing a nylon filter with a pore size of 3 μm (Millipore, Billerica, USA). The tubes were placed upside down in a glass beaker containing 200 ml of the same isotonic phosphate buffer. Sodium azide (0.05%) was added to prevent bacterial growth during the release experiment. The temperature of the buffer was 37 °C and the tubes were rotated at a speed of 50 rpm. During a time span of 270 h (∼10 times the half-life of Ho-166), the release system was dismantled periodically for release measurements. The release of holmium was determined by comparing the measured activity of Ho-166m in the glass beaker versus the activity of the microspheres in the tubes using a low-background γ-counter (Tobor, Nuclear Chicago, USA). Ho-166m is a metastable isotope formed in a small fraction (7 ppm of the total amount of newly formed isotopes) during activation of Ho-165. Due to the low concentration of Ho-166m in the samples and its long half-life (∼1200 yr), the actual activity of Ho-166m is very low. However, after the decay of Ho-166 (with a half-life of 26.8 h), Ho-166m is the only remaining isotope and can therefore still be reliably detected as demonstrated previously (Seppenwoolde et al., 2004).

3. Results and discussion

3.1. Preparation of microspheres

Ho-PLLA-MS with 17.0 ± 0.5% (w/w) of holmium, corresponding with a loading of the HoAcAc complex of ∼50% (w/w), were prepared according to the standard preparation method (Nijsen et al., 1999). The microspheres have a rather wide-ranging size distribution (10–60 μm) (Fig. 3). Sieving with either method A or method B was very effective in narrowing down the particle size distribution: more than 97% (v/v) of the microspheres had a size of between 20 and 50 μm after sieving, which is the desired size for an optimal targeting (Nijsen et al., 2002b). Sieving method A is preferred since this method is less time consuming than method B (10 min versus 45 min), and less water is needed for the total sieving procedure (1 l versus 8 l). The total mass of microspheres was about 4 g after sieving.

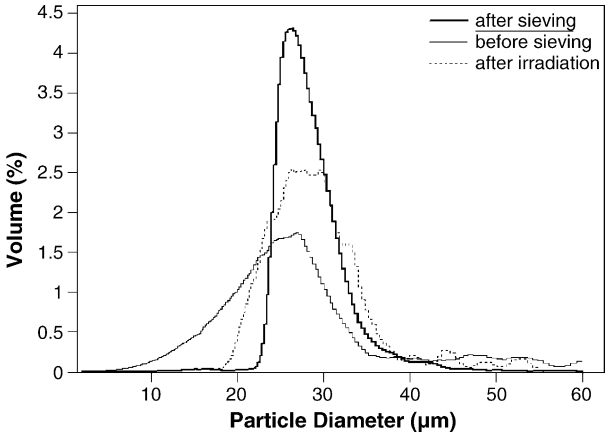

Fig. 3. Typical population distribution of microspheres before and after sieving, and after irradiation in a nuclear reactor.

Since higher residues of dichloromethane are allowed by the ICH-guidelines (B'Hymer, 2003), this solvent was also tested. The particle size distribution and yield were equal to Ho-PLLA-MS prepared with chloroform. However, the holmium content of the microspheres prepared using dichloromethane was 9%, which is much lower than in the microspheres that were prepared with chloroform (17%). Previous studies have shown that the solubility of the holmium-complex in PLLA is influenced by the method of solvent removal. HoAcAc is less soluble in films (up to 8%, prepared by the quick evaporation of chloroform) than in microspheres (17%, prepared by the solvent evaporation method as described in Section 2.2). The difference in HoAcAc loading between microspheres prepared by either chloroform or dichloromethane may be caused by the higher water solubility (leading to a fast solvent extraction) and the lower boiling point (leading to a fast solvent evaporation) of dichloromethane, when compared with chloroform. A high holmium content of the microspheres is required in order to achieve therapeutic amounts of radioactivity in a relatively small portion of microspheres (500–800 mg). The low holmium content of the microspheres prepared using dichloromethane does not meet this requirement and makes them unsuitable for clinical application. A small amount of microspheres will result in the lodging of the microspheres in and around the tumour, whilst sparing healthy liver tissue (Nijsen et al., 2001a,b), and will prevent filling of the supply vessels of the liver (Seppenwoolde et al., 2004) which will cause unnecessary damage to healthy liver tissue. Furthermore, in contrast to microspheres prepared with chloroform, Ho-PLLA-MS which were prepared with dichloromethane had a very irregular shape (Fig. 4). It has previously been reported that the use of dichloromethane can lead to the formation of microspheres with an irregular shape (Wang et al., 1997; Chung et al., 2001). The consequences of this irregular microsphere morphology in terms of aggregation of the microspheres during administration in the catheter or blood vessels are not known.

HoAcAc obtained from a commercial source (Sigma–Aldrich) is unsuitable for the preparation of microspheres (five attempts were made). Visual differences were clearly observed between the HoAcAc obtained form Sigma–Aldrich (small orange coloured rhombic crystals) and HoAcAc as synthesised

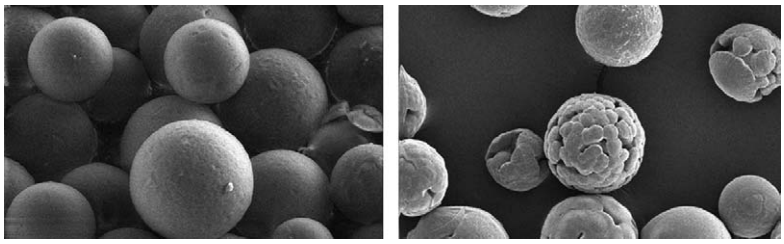

Fig. 4. SEM pictures of Ho-PLLA-MS produced with chloroform (left) and with dichloromethane (right).

by ourselves (pink needle-shaped crystals). During the washing step with 0.1 M HCl severe flocculation of the particles occurred and no microspheres could be obtained. Previous studies have demonstrated that the synthesis of HoAcAc is a delicate procedure (Nijsen et al., 1999; Kooijman et al., 2000), which can in some cases lead to the formation of impure material. These impurities are probably the cause of the unsuccessful preparation of Ho-PLLA-MS with HoAcAc obtained form Sigma–Aldrich.

Fig. 5 demonstrates that without temperature control, the temperature of the water/chloroform mixture decreased tremendously (from ∼20 to ∼8 °C), and remained at this temperature. As a result the chloroform could not be quantitatively evaporated within 40 h. Both types of temperature control (see Fig. 1) were sufficient to maintain the temperature at room temperature, and both resulted in the quantitative evaporation of chloroform from the water phase after 40 h. However, use of an open water bath (situation B, Fig. 1) proves very susceptible to bacterial contamination and is, therefore, undesirable in a GMP-production process.

3.2. Pharmaceutical quality of microspheres

No colony forming units (CFU) (aerobe bacteria, anaerobe bacteria and fungi) could be found in the examined microsphere batches. The production of Ho-PLLA-MS is a very time consuming process (the total time is around 4 days) and in several production steps water is used, thus rendering the process very vulnerable to bacterial contamination and growth. However, we introduced several germ-reducing steps in the production process (see Section 2.2), which are:

- boiling of the PVA solution;
- flushing with filtered (0.22 μm HEPA-filter) nitrogen gas over the stirred water/chloroform mixture;
- washing the microspheres with 0.1 M HCl;
- drying of the microspheres for 5 h at 70 °C under vacuum.

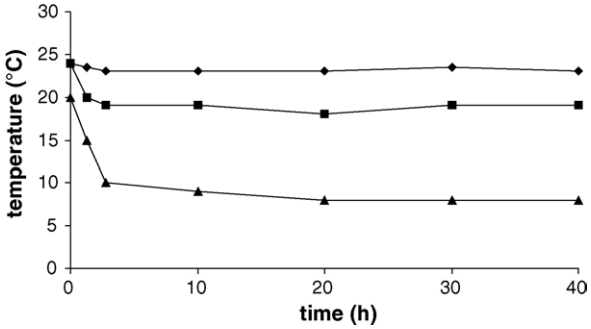

Fig. 5. Temperature monitoring during the solvent evaporation process (40 h). (▲) No temperature control, (■) set up A, (♦) set up B.

These germ-reducing steps appear to be sufficient to obtain CFU-free microspheres. In view of the fact that microspheres are subsequently neutron irradiated with a very high dose of radiation, the sterility of the microspheres that are administered to a patient can be guaranteed.

The endotoxin level detected ranged from 3.7 to 6.1×10^{-4} EU/mg microspheres. The limit for parenteral drugs is 5.0 EU/kg body weight (Ph. Eur., 2002). The maximum amount of endotoxins allowed to be administered to a patient of 70 kg is 350 EU. If, as in our case, 800 mg of Ho-PLLA-MS is administered to patients, the total amount of endotoxins (∼0.5 EU) is well below the given limits.

With respect to their residual solvent levels, Ho-PLLA-MS were in compliance with the ICH-guidelines (B'Hymer, 2003). No residual solvents were detected in the three batches of neutron irradiated Ho-PLLA-MS. The absence of chloroform in microspheres, used during the production process, is caused by an intensive drying process (70 °C under vacuum) and the subsequent neutron irradiation of Ho-PLLA-MS (Zielhuis et al., 2005c).

3.3. Radiochemical stability

Although neutron irradiation resulted in a very high radiation dose to the Ho-PLLA-MS (Nijsen et al., 2002a), only minor changes in the particle size distribution were observed. After irradiation, more than 94% (v/v) of the microspheres had the desired particle size of between 20 and 50 μm (Fig. 3).

Moreover, neutron irradiated Ho-PLLA-MS were very stable. After 24 h only 0.3 ± 0.1% of Ho had been released from the microspheres in the buffer. No further release of Ho was observed until the end of the experiment (270 h, ∼10 times the half-life of Ho-166). It had previously been demonstrated that holmium and PLLA interact with each other (Nijsen et al., 2001a,b). These interactions are probably the cause of the high stability of neutron irradiated Ho-PLLA-MS.

4. Conclusion

In this paper the GMP-production of Ho-PLLA-MS is described. It has been demonstrated that it is possible to prepare

microspheres by a solvent evaporation process on a gram-scale. The pharmaceutical characteristics (residual solvents, CFU and endotoxins) of the microspheres are in compliance with the requirements of the European Pharmacopoeia. Furthermore, neutron-irradiated microspheres have a high radiochemical stability and retain their size and shape.

In conclusion, radioactive Ho-PLLA-MS which are produced as described in this paper can be clinically applied giving due consideration to their pharmaceutical quality.

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