Nuclear Medicine and Biology 71 (2019) 11–18

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

Blood and urine analyses after radioembolization of liver malignancies with [^{166}Ho]Ho-acetylacetonate-poly(L-lactic acid) microspheres

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ARTICLE INFO

Article history:
Received 28 September 2018
Received in revised form 17 March 2019
Accepted 23 March 2019

Keywords:
SIRT
Holmium
Radioisotope
Blood
Urine

ABSTRACT

Background: [^{166}Ho]Ho-acetylacetonate-poly(L-lactic acid) microspheres were used in radioembolization of liver malignancies by intra-arterial administration. The primary aim of this study was to assess the stability and biodistribution of these microspheres.

Materials and methods: Peripheral blood and urine samples were obtained from two clinical studies. Patient and in vitro experiment samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS), gamma-ray spectroscopy, light microscopy, Coulter particle counting, and high performance liquid chromatography (HPLC).

Results: The median percentage holmium compared to the total amount injected into the hepatic artery was 0.19% (range 0.08–2.8%) and 0.32% (range 0.03–1.8%) in the 1 h blood plasma and 24 h urine, respectively. Both the blood plasma and urine were correlated with the neutron irradiation exposure required for [^{166}Ho]Ho-AcAc-PLLA microsphere production ($\rho = 0.616$, $p = 0.002$). After a temporary interruption of the phase 2 clinical study, the resuspension medium was replaced to precipitate [^{166}Ho]Ho^{3+} pre-administration using phosphate. The in vitro near-maximum neutron irradiation experiments showed significant [^{166}Ho]Ho-AcAc-PLLA microsphere damage.

Conclusion: The amount of holmium in the peripheral blood and urine samples after [^{166}Ho]Ho-AcAc-PLLA microsphere intrahepatic infusion was low. A further decrease was observed after reformulation of the resuspension solution but minimization of production damage is necessary.

1. Introduction

Radioembolization using microspheres containing yttrium-90 (^{90}Y) injected directly into the hepatic artery delays the progression of liver malignancies [1,2]. The addition of [^{90}Y]Y-resin radioembolization to fluoropyrimidine-based chemotherapy was found to significantly improve median progression-free survival in the liver by 7.9 months in patients with metastatic colorectal cancer [3]. Furthermore, in patients with primary liver cancer, ^{90}Y-labelled resin radioembolization was better tolerated than targeted therapy with the tyrosine kinase inhibitor sorafenib [4]. However, to date, no survival benefit has been established in phase 3 studies [3–7].

[^{166}Ho]*Ho*-acetylacetonate-poly(L-lactic acid) microspheres ([^{166}Ho]Ho-AcAc-PLLA microspheres) have been studied in patients with liver malignancies of various origins as an option for improving the dosimetry for more personalized treatment [8–13]. ^{166}Ho differs in its decay from ^{90}Y. ^{166}Ho emits therapeutic beta-particles of 1.77 MeV and 1.85 MeV and it has a physical half-life of 26.8 h. In contrast, ^{90}Y has a longer physical half-life of 64.0 h and it emits higher energy therapeutic beta-particles (2.28 MeV). Holmium distribution can be imaged with magnetic resonance imaging (MRI) or single photon emission computed tomography (SPECT) due to the low energy 81 keV gamma emission [8–10,14]. ^{90}Y has no gamma emission, but it can be detected using Bremsstrahlung-emission SPECT and positron emission tomography (PET), because of the low level of positron emission [15].

The present study, aimed to assess the stability of [^{166}Ho]Ho-AcAc-PLLA microspheres used for radioembolization in a clinical setting. Therefore, blood and urine samples of patients from the Holmium Embolization Particles for Arterial Radiotherapy (HEPAR) phase 1 and phase 2 studies were analyzed. Furthermore, the effect of the changed composition of the resuspension medium on the [^{166}Ho]Ho-AcAc-PLLA microspheres was also analyzed.

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https://doi.org/10.1016/j.nucmedbio.2019.03.001

2. Materials and methods

2.1. Ho-AcAc-PLLA microspheres preparation

The preparation of Ho-AcAc-PLLA microspheres was previously described [16–18]. In brief, 10 g of $HoCl_3.6H_2O$ (Metall Rare Earth Ltd., Shenzhen, China) was dissolved in 100 mL of sterile water (Fresenius Kabi, Bad Homburg, Germany). This solution was then added to a solution containing 180 g of acetylacetone (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands) and 1080 mL of sterile water, pH 8.5 (adjusted with NH_4OH, Spruyt Hillen, IJsselstein, the Netherlands) and homogenized by stirring for 1 min. Subsequently, Ho-AcAc crystals were formed after the solution stood at room temperature for 15 h. After washing the Ho-AcAc crystals three times with sterile water, they were air-dried at 50 °C for 48 h. The Ho-AcAc crystals (10 g) and PLLA (6 g) (Purac Biochem, Gorinchem, the Netherlands) were then dissolved in chloroform (186 g) (Merck, Darmstadt, Germany) and were added to a 1.0 L of 2% polyvinylalcohol solution (PVA) (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands). The chloroform of this oil-in-water emulsion was evaporated under continuous stirring (IKA Eurostar power digi-visc stirrer) in baffled beakers using a nitrogen flow at 25 °C for 40–72 h. No residual chloroform could be detected in the microspheres after neutron irradiation [16]. The resulting Ho-AcAc-PLLA microspheres were washed three times with 0.1 Mol/L HCl (Merck, Darmstadt, Germany) and three times with sterile water, they were then sieved to obtain the desired size of 15–60 μm using an electronic sieve vibrator (TOPAS EMS755, Dresden, Germany) and an ultrasonic processor (Hielscher UP200S, Teltow, Germany). Finally, the Ho-AcAc-PLLA microspheres were air-dried (50 °C, 24–48 h) and vacuum dried (70 °C, 7 h)."

2.2. Neutron irradiation

The Ho-AcAc-PLLA microspheres were neutron irradiated in a nuclear reactor (thermal flux: $1.4–1.8 \times 10^{20}$ neutrons m^{-2} h^{-1}, Reactor Institute Delft, Delft, The Netherlands). This resulted in holmium-166 and small amounts of holmium-166m due to the thermal neutron cross sections of 64 barn and 3.1 barn, respectively [17]. A typical neutron irradiation of 540 mg Ho-AcAc-PLLA microspheres with a thermal neutron flux of 1.6×10^{20} neutrons m^{-2} h^{-1} for 4.0 h leads to approximately 10.0 GBq [^{166}Ho]Ho-AcAc-PLLA microspheres and 1.3 kBq [^{166m}Ho]Ho-AcAc-PLLA microspheres at the end of neutron irradiation. The relatively small amount of ^{166m}Ho with a physical half-life of 1200 years was considered negligible both in patients and in waste.

2.3. Sterility and apyrogenicity

The equipment used for preparing the Ho-AcAc-PLLA microspheres were steam sterilized at 121 °C for 30 min at the Central Sterilization Department of the University Medical Center Utrecht. Furthermore, preparation was carried out in a class-D clean room (Ph. Eur., 2002), and it consisted of the following germ-reducing steps: boiling of the PVA solution, flushing with filtered (0.22 m HEPA-filter) nitrogen gas over the stirred water/chloroform mixture, washing the microspheres with 0.1 mol/L HCl, and drying them for 5 h at 70 °C under a vacuum condition [18].

After preparation of the Ho-AcAc-PLLA microspheres, bioburden and endotoxin tests were conducted for each batch before neutron irradiation to confirm sterility and apyrogenicity using the European Pharmacopoeia 5.0 methods and regulations 2.6.12, 2.6.13 and 2.6.14 [19] (carried out by Bactimm B.V., Nijmegen, The Netherlands). Only the batches that met these requirements were used. During neutron irradiation, the Ho-AcAc-PLLA microsphere samples received an additional excessive gamma dose for final sterilization (>25 kGy) as described previously [18,20]. This samples spiked with Bacillus pumilis spores. Ten samples were neutron irradiated with 0.9×10^{20} thermal neutrons m^{-2} and ten control samples were not neutron irradiated. All samples were tested for bacterial contamination by a certified company and only the neutron irradiated Ho-AcAc-PLLA microspheres were sterile (Bactimm B.V., Nijmegen, The Netherlands).

2.4. Quality controls and in vitro experiments

After neutron irradiation [^{166}Ho]Ho-AcAc-PLLA microspheres were resuspended using a medium containing sterile water for injection with ethanol 10% (v/v) and 2% w/v of polyoxyethylene-polyoxypropylene copolymer (Pluronic® F-68, Sigma-Aldrich® Chemie B.V., Zwijndrecht, The Netherlands). The HEPAR 2 study was temporarily halted because of toxicity concerns raised from new animal studies. Clinical outcomes [8,10] and additional SPECT-CT analyses were evaluated and the microspheres were considered to be safe. In addition, brief stability tests using the resuspension medium containing 116 mM phosphate buffer (pH 7.2) and 2% w/v Pluronic® F-68 were performed for these microspheres that are classified as a medical device. The previous, in combination with the desire to provide palliative treatment to these patients, lead to the re-start of the study without extensive and time-consuming new tests.

Batches of both Ho-AcAc-PLLA and [^{166}Ho]Ho-AcAc-PLLA microspheres were visually inspected for irregularities and damage by light microscopy (Eclipse E200 Microscope, Nikon, Japan). In addition, particle size and volume distribution analyses were performed by Coulter counting (Coulter counter III, Beckman, USA). An average diameter of 30 ± 5 μm and a particle volume of >97% between 15 and 60 μm was required.

In additional in vitro experiments the amount ^{166}Ho, Ho and acetylacetonate in the resuspension media was measured at 1, 3, 6 and 24 h to assess the stability. For this, [^{166}Ho]Ho-AcAc-PLLA microspheres were centrifuged for 5 min at 500*g* and the 200 μL taken from the supernatant was centrifuged again for 5 min at 500*g*. Subsequently, 150 μL of the supernatant was analyzed for ^{166}Ho, Ho, and AcAc.

2.5. Analyses

2.5.1. ICP-MS

All blood plasma and urine samples were digested in twice the sample volume of 65% HNO_3 at room temperature for 3 days and subsequently diluted to 14% (v/v) HNO_3. The Ho content was only determined in the blood plasma, as blood plasma is better digested than whole blood and consequently results in more robust ICP-MS results. Calibration and background samples were digested identically. Blood plasma and urine samples from untreated instead of treated patients were used for standard addition of an ICP-MS holmium standard (Ho_2O_3 in HNO_3 2–3%, Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands). Ho content was determined using a PerkinElmer NEXON 350D ICP-MS (PerkinElmer, Groningen, The Netherlands). A Microflow PFA Nebulizer (PE N8145101) was used for sample introduction into the spray chamber of the ICP-MS. The spray chamber itself was a Quartz Cyclonic spray chamber (PE Revision A, N8145013). Furthermore, the ICP-MS was equipped with a platinum sample cone and a platinum skimmer cone. The NEXION 350D was operating at a forward power of 1600 W and the argon gas flow settings were: plasma gas flow 14 L/min, auxiliary gas flow 1.2 L/min and nebulizer flow 1.1 L/min. Data acquisition was done in standard mode with holmium measured at *m/z* 165 and rhodium as an internal standard at m/z 103. The mean difference between calculated standard and ICP-MS measurement in urine and blood plasma was 1.00 ± 0.03% and was used to calculate the final ICP-MS measurements.

2.5.2. Gamma counting

^{166}Ho activity and background were measured using a gamma counter (15 min per sample, Wizard 3, 1480 automatic Gamma counter, PerkinElmer Life and Analytical Sciences, USA). Calibration was

performed using ^{166}Ho samples measured in a validated dose calibrator (VDC-404; Veenstra Instrumenten B.V., Joure, The Netherlands).

2.5.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC), Thermo Scientific Dionex Ultimate 3000, Thermo Scientific, Waltham, USA) with UV detecting was used to measure acetylacetonate using an Acclaim 120 stainless steel C18 column (120 A, 3 μm, 4.6 × 150 mm). Elution (0.5 mL/min) was performed using 70% methanol (HPLC grade, VWR International, The Netherlands) and 30% of 0.1% trifluoroacetic acid in H_2O (Biosolve B.V., Valkenswaard, The Netherlands) in 10 min. The retention time of acetylacetonate was 3.7 ± 0.2 min. Calibration samples were prepared 0–1000 mg/L for quantifications (Sigma-Aldrich® Chemie B.V., Zwijndrecht, The Netherlands).

2.6. Patient studies

Patients with various liver malignancies were treated by intra-arterial injection of in total 600 mg [^{166}Ho]Ho-AcAc-PLLA microspheres in the HEPAR 1 (NCT01031784) and HEPAR 2 studies (NCT01612325) [8,10]. The specific activity of the [^{166}Ho]Ho-AcAc-PLLA microspheres varied because of the dose escalation design HEPAR 1 and the liver size in the HEPAR 2. All patients provided written informed consent. The sample collections were approved also by the Medical Research Ethics Committee of the University Medical Center Utrecht in The Netherlands.

Three intra-arterial injection procedures in the liver were performed per patient. The first two procedures were primarily for safety and treatment optimization reasons, the third one was the actual treatment. First, ^{99m}Tc[Tc]-macroaggregates (150 MBq, TechnescanLyoMAA, Mallinckrodt Medical B.V, The Netherlands) were injected 1–2 weeks before treatment planning. Secondly, low apparent specific activity [^{166}Ho]Ho-AcAc-PLLA microspheres (250 MBq per 60 mg) were injected prior to the planned treatment the same day. Thirdly, 540 mg therapeutic [^{166}Ho]Ho-AcAc-PLLA microspheres (6.3–24.8 GBq/g) was administrated. After both safety procedures a SPECT-CT was done within 3 h, post-treatment SPECT-CT and MRI were carried out within 3–6 days, as described previously [8,10].

3. Peripheral blood and urine samples

Blood samples were taken at various timepoints after administration of the therapeutic dose. Urine was cumulatively collected at several time intervals after therapy. 1 mL of whole blood was centrifuged for 10 min at 500*g* to precipitate cells and cell fragments. 0.50 mL of blood plasma was taken for ICP-MS analyses. The ^{166}Ho activity of the blood and urine samples was only measured after the temporary halt of the HEPAR 2 study. In a subgroup of 5 patients, also the ^{166}Ho activity distribution between whole blood and blood plasma was measured in 1, 3, 6 and 24 h samples. The average 64.3% activity in blood plasma of these 20 whole blood samples was used to calculate the blood plasma activities for comparison with ICP-MS measurements.

4. Statistical analysis

Continuous data are presented as the mean ± standard deviation (SD) if normally distributed and as the median (min-max) if skewed (Shapiro-Wilk test). Categorical data are presented as numbers and proportions. Differences between the treatment characteristics and analytical samples were evaluated by the $\chi 2$ test for categorical data and by the Student's *t*-test for continuous data if normally distributed. The Mann-Whitney *U* test was used if the data were skewed. Correlations were evaluated with Pearson (r) if normally distributed or Spearman rank (ρ) if data were skewed. The data were registered in a dedicated database. The statistical analysis was conducted using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The significance level was set at $p \leq 0.05$.

5. Results

In total 23 patients were treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 2 mL of 2% Pluronic® F-68 (w/v) in 10% ethanol (v/v). The Ho content of the blood plasma and the urine samples as measured by ICP-MS is presented in Table 1 and Fig. 1. A relatively low amount of Ho was detected in both blood plasma at 1 h (median 0.19% of the injected activity; range 0.08–2.8) and cumulative 24 h urine (median 0.32%; range 0.03–1.8%). A rapid Ho decrease in both blood plasma and urine was measured over time but Ho remained detectable for up to several months (Table 1).

Irradiation of 540 mg Ho-AcAc-PLLA microspheres with a median number of 4.0×10^{20} thermal neutrons/m^2 (range 1.5–8.9) resulted in a median apparent specific activity of 14.4 GBq/g of therapeutic [^{166}Ho]Ho-AcAc-PLLA microspheres at the end of neutron irradiation (range 5.0–28.7). The median time between resuspension and patient administration was 2.0 h (range 0.5–6.0). The median injected activity was 5.2 GBq (range 3.4–11.3). A correlation between the irradiation and the amount of Ho in blood plasma and urine samples ($\rho = 0.57$ ($p < 0.01$) and $\rho = 0.62$ ($p < 0.01$), respectively) was detected for up to 24 h (Fig. 2). In addition, a relation was established between Ho in blood and Ho in urine ($\rho = 0.87$; $p \leq 0.01$).

After substitution of the resuspension medium, a total of 30 patients were treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 2 mL of 2% Pluronic® F-68 (w/v) in 116 mM phosphate pH 7.2. The median number of thermal neutrons and resuspension time were 5.4×10^{20} neutrons/m^2 (range 3.2–9.1) and 1.8 h (range 0.2–8.4), respectively, resulting in a median apparent specific activity of 18.7 GBq/g (range 10.9–28.7) at the end of irradiation. The median injected activity was 6.4 GBq (range 5.0–13.2). The median blood plasma values at 1 h and cumulative urinary 24 h excretion were 0.09% (range 0.00–0.61) and 0.06% (range 0.01–0.40) of the injected activity, respectively (Table 2 and Fig. 1). This was a significant decrease ($p < 0.05$) in comparison with the previously used resuspension medium.

The ^{166}Ho content was measured in the last 30 patients (Table 3). The median blood plasma values at 1 h and cumulative urinary 24 h excretion were 0.003% (range 0.000–0.032) and 0.003% (range

Table 1
The median percentage of the infused amount of Ho as determined by ICP-MS in blood plasma and urine from patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 10% ethanol (v/v) 2% Pluronic® F-68.

Blood plasma	N (of total)	μg/L		%	
		Median	Min – Max	Median	Min – Max
1 h	8 (15)	57.0	33.0–529.8	0.19	0.08–2.81
3 h	21 (23)	63.4	2.1–935.0	0.15	0.00–2.43
6 h	22 (23)	46.0	1.3–626.5	0.14	0.01–1.63
24 h	21 (23)	18.8	0.5–259.8	0.06	0.00–0.60
48 h	13 (15)	7.1	0.3–59.6	0.02	0.00–0.18
7 weeks	15 (15)	1.4	0.1–26.5	0.00	0.00–0.08
13 weeks	13 (15)	1.7	0.3–13.2	0.01	0.00–0.04
Urine	**N (of total)**	**μg**		**%**	
		Median	Min – Max	Median	Min – Max
0–3 h	18 (23)	110.7	12.1–892.4	0.13	0.01–1.19
3–6 h	22 (23)	88.0	1.4–692.6	0.08	0.00–0.72
6–24 h	23 (23)	67.7	3.2–469.0	0.07	0.00–0.54
24–48 h	15 (15)	20.1	0.7–114.4	0.02	0.00–0.10
7 weeks	15 (15)	5.3	2.6–19.2	0.01	0.00–0.02
13 weeks	15 (15)	4.5	1.6–18.3	0.01	0.00–0.02
Cumulative 0–24 h	23 (23)	340.5	25.4–1437	0.32	0.03–1.80

N: number, %: percentage of injected amount.

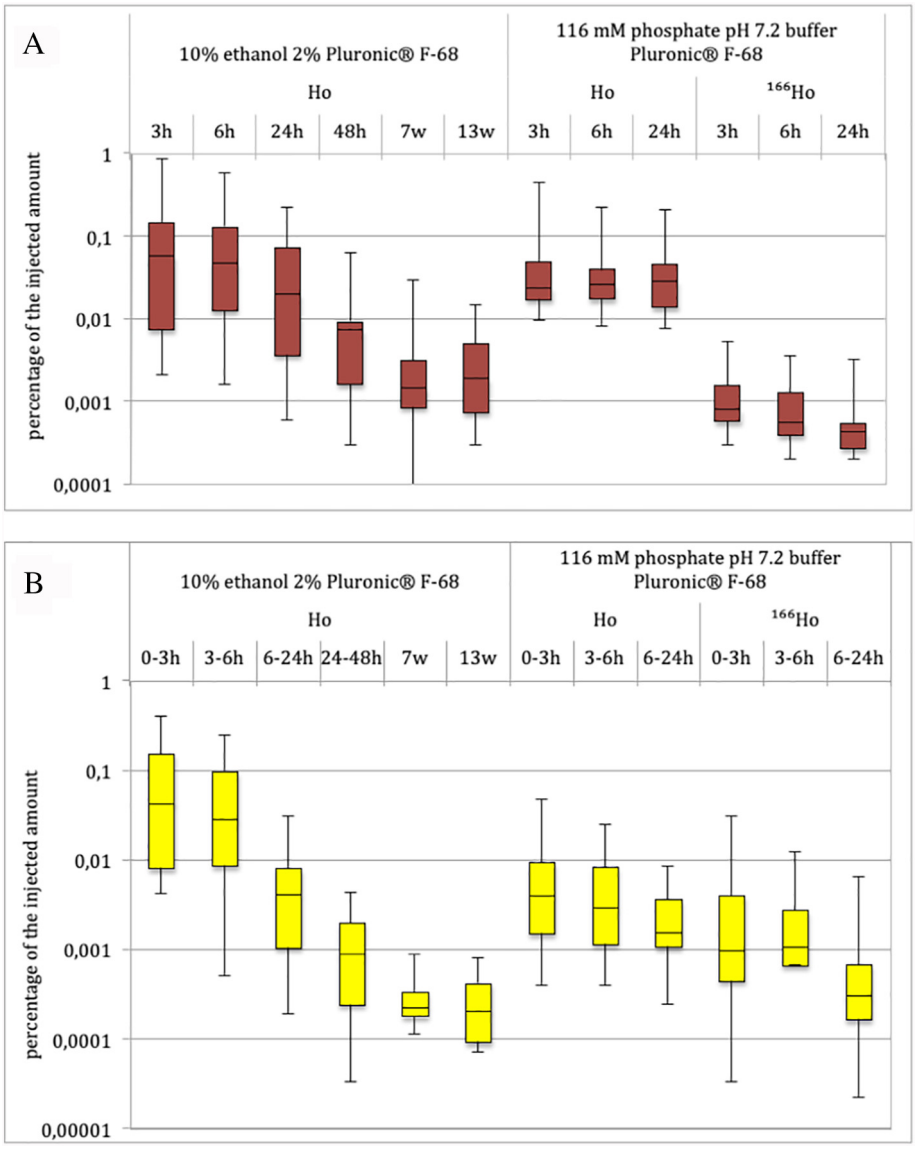

Fig. 1. Amounts of Ho and ^{166}Ho in blood plasma (A) and urine (B) of patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres as the percentage of the injected amount. Amounts of Ho and ^{166}Ho in blood plasma (A) and urine (B) of patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres as the percentage of the injected amount. The number of measurements is represented in Tables 1–3.

0.000–0.022) of the injected activity, respectively. A significant correlation was observed between the injected activity and the percentage of Ho and ^{166}Ho in the 24 h cumulative urine ($\rho = 0.38$; $p = 0.04$ and $\rho = 0.60$; $p < 0.01$), respectively. In the blood, a trend was observed between the injected activity and both the Ho and ^{166}Ho content at 1, 3, 6 and 24 h. The mean ratio between Ho and ^{166}Ho was 0.03 ± 0.03 in blood and 0.39 ± 0.27 in urine; both ratios were significantly different from 1.0 ($p < 0.01$), so no equilibrium existed between both isotopes.

Quality control analyses of especially the highest neutron irradiated [^{166}Ho]Ho-AcAc-PLLA microspheres for patients showed that the change of resuspension media to phosphate buffered solution altered the microscopic appearance (data not shown). The visual analysis revealed damage consisting of small fragments and, occasionally, dented surfaces. In comparison with the ethanol-containing resuspension medium, the phosphate buffered solution contained additional $HoPO_4$ precipitates both at the [^{166}Ho]Ho-AcAc-PLLA microsphere surface and in the resuspension medium. This was confirmed by the occasionally strongly increased number of small particles, as determined by particle size distribution analysis (Fig. 3). The maximum small particle volume was within pre-set limitations (0–3% v/v) for use in clinical trials, and subsequently, these therapeutic doses were administrated.

To verify these observations in vitro, [^{166}Ho]Ho-AcAc-PLLA microspheres were analyzed using a previously used near-maximum neutron irradiation of 8.9×10^{20} m^{-2} thermal neutrons and incubation times of 1, 3, 6 and 24 h (Table 4). The visual analysis of these [^{166}Ho]Ho-AcAc-PLLA microspheres revealed similar damage to those used for study patients. After near-maximum irradiation, the mean cumulative leakage of ^{166}Ho and Ho at 1 h after resuspension in a 2% Pluronic® F-68 (w/v) 10% ethanol (v/v) medium was as high as 15.7 ± 1.7% and 15.0 ± 2.5%, respectively (Table 4). Furthermore, the leakage increased with longer incubation time. The use 2% Pluronic® F-68 (w/v) in 116 mM phosphate pH 7.2 medium resulted in the decreased supernatant presence of ^{166}Ho and Ho to 0.09 ± 0.01% and 0.18 ± 0.14%, respectively (Table 4). Control experiments showed complete precipitation of [^{166}Ho]Ho^{3+} in the 116 mM phosphate pH 7.2 resuspension medium, in contrast to its supernatant presence when using 2% Pluronic® F-68 (w/v) 10% ethanol (v/v).

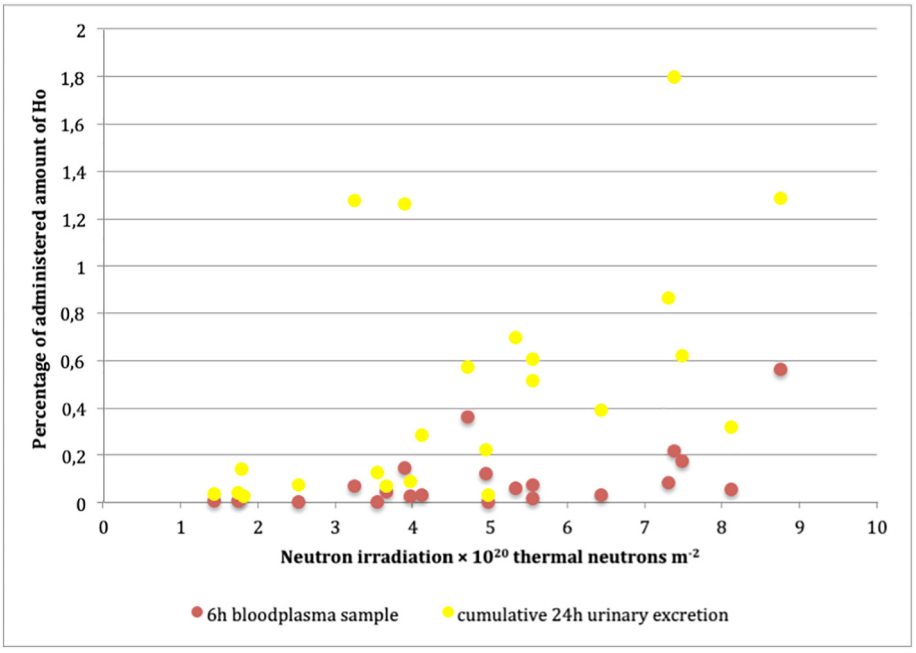

Fig. 2. The influence of neutron irradiation on the amount of holmium-165 in blood plasma and urine of patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 10% ethanol (v/v) 2% Pluronic® F-68. Scatterplot showing the influence of neutron irradiation on the amount of holmium-165 in blood plasma (red dots) and urine (yellow dots) of patients ($n = 23$) treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 10% ethanol (v/v) 2% Pluronic® F-68.

A yellowish discolouration of both media was already observed within 1 h after resuspension. HPLC analysis showed acetylacetonate leakage corresponding to 3.0–10.0% of the total amount initially incorporated in the [^{166}Ho]Ho-AcAc-PLLA microspheres (Table 4). In addition, at least one other absorption peak was detected but the probable presence of radiolytic leachables was not investigated in further detail (data not shown).

6. Discussion

The HEPAR 1 and 2 studies were the first clinical experiences with ^{166}Ho radioembolization. The blood and urine samples were part of the study design to investigate the in vivo stability of [^{166}Ho]Ho-AcAc-PLLA microspheres. The total amount of holmium in the blood plasma and urine samples for both resuspension fluids used was relatively low and declined quickly over time (Tables 1–3, Fig. 2). These relatively low blood and urine measurements are consistent with reported results from ^{166}Ho-SPECT, performed three to seven days after therapy [8,10,14]. Up to 13 weeks after treatment, small amounts of Ho were detected in the blood and urine samples (Table 2). Therefore, the release appears to be biphasic consisting of a rapid initial phase and a slower phase. The latter phase could be due to slow in vivo biodegradation of the microspheres as previously reported [20–22].

The neutron irradiation of [^{166}Ho]Ho-AcAc-PLLA microspheres was correlated with the amount of Ho and ^{166}Ho in the collected blood plasma and urine samples (Fig. 1). This damage is probably caused by gamma heating (free radicals from gamma radiation plus a temperature increase) and fast neutrons [20,23,24]. Concerns about the stability of [^{166}Ho]Ho-AcAc-PLLA microspheres and the absence of a chemical equilibrium between ^{166}Ho and Ho were raised during the clinical HEPAR 2 study, resulting in a temporary halt. In other words, thermal neutron capture causes ^{166}Ho recoil that can release from the acetylacetonate complex, and ^{166}Ho decay causes reactive oxygen species during incubation in a resuspension medium [25–27]. Therefore, ^{166}Ho was also measured after resuming the HEPAR 2 study, because this is more important than Ho from a toxicity point of view, as a median lethal dose of

Table 2
The median amount of Ho as determined by ICP-MS in blood plasma and urine of patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 116 mM phosphate 2% Pluronic® F-68 pH 7.2.

Blood plasma	N (of total)	μg/L		%	
		Median	Min – Max	Median	Min – Max
1 h	29 (30)	27.8	0.0–219.8	0.087	0.000–0.606
3 h	29 (30)	10.8	0.8–379.8	0.035	0.000–1.220
6 h	29 (30)	15.8	0.0–239.8	0.046	0.003–0.609
24 h	25 (30)	16.8	0.0–229.8	0.049	0.000–0.571
Urine	**N (of total)**	**μg**		**%**	
		Median	Min – Max	Median	Min – Max
0–3 h	27 (30)	8.0	0.0–123.9	0.008	0.000–0.120
3–6 h	36 (30)	3.0	0.1–68.2	0.004	0.000–0.066
6–24 h	30 (30)	15.6	0.0–129.9	0.015	0.000–0.126
0-24 h	30 (30)	60.1	12.0–415.4	0.059	0.011–0.403

N: number, %: percentage of injected amount.

Table 3
The median amount and range of ^{166}Ho as measured by gammadetection in blood plasma and urine of patients treated with [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 116 mM phosphate pH 7.2, 2% Pluronic® F-68.

Blood plasma	N (of total)	Bq/mL		%	
		Median	Min – Max	Median	Min – Max
1 h	25 (30)	34.8	5.7–573.1	0.003	0.000–0.032
3 h	26 (30)	23.7	6.7–213.6	0.003	0.001–0.017
6 h	26 (30)	16.0	4.1–105.5	0.002	0.006–0.011
24 h	23 (30)	9.5	2.4–30.8	0.001	0.006–0.010
Urine	**N (of total)**	**kBq**		**%**	
		Median	Min – Max	Median	Min – Max
0–3 h	25 (30)	175.1	3.8–6867	0.003	0.000–0.090
3–6 h	23 (30)	156.4	1.6–2274	0.003	0.000–0.036
6–24 h	25 (30)	171.1	14.8–1211	0.005	0.000–0.024

N: number, %: percentage of total injected activity calculated to the time of injection.

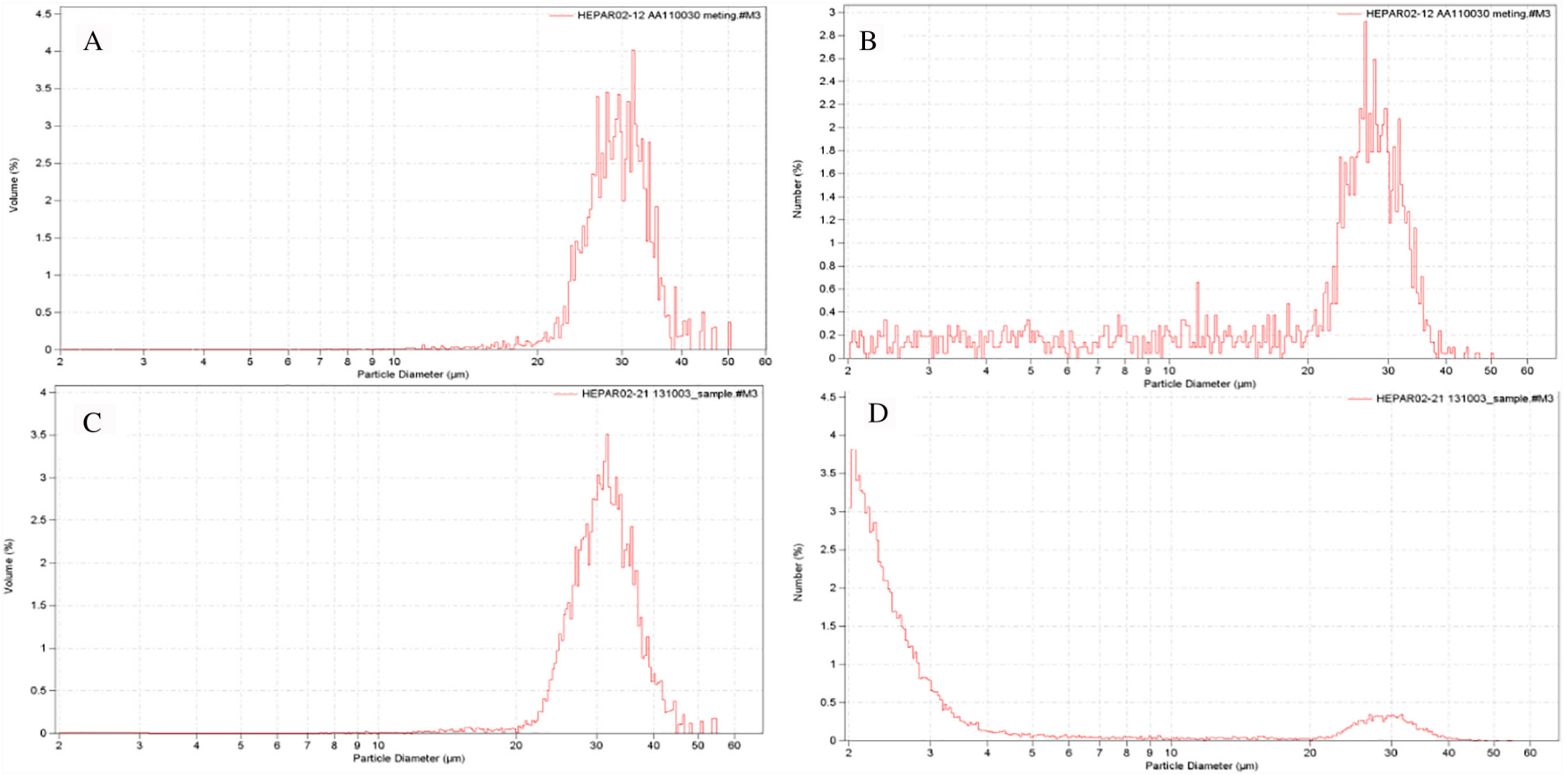

Fig. 3. Size distribution plots of [^{166}Ho]Ho-AcAc-PLLA microspheres after irradiation. Size distribution plots of [^{166}Ho]Ho-AcAc-PLLA microspheres after approximately 8.9×10^{20} m^{-2} thermal neutrons and 1 h in suspension showing the difference in size distribution. (A-B) [^{166}Ho]Ho-AcAc-PLLA microspheres in the 10% ethanol and (C-D) 116 mM phosphate pH 7.2 buffered solution. Showing a similar particle volume (A, C) but a difference in particle size distribution with a large amount of small holmium phosphate particles (B, D).

Table 4
Mean ($n = 3$) percentages of ^{166}Ho, Ho and acetylacetonate in the supernatant after centrifugation of [^{166}Ho]-Ho-AcAc-PLLA microspheres in two different media.

	10% ethanol 2% Pluronic® F-68			116 mM phosphate pH 7.2 buffer Pluronic® F-68		
Time	^{166}Ho	Ho	AcAc	^{166}Ho	Ho	AcAc
1 h	15.7 ± 1.7%	15.0 ± 2.5%	3.0 ± 0.2%	0.09 ± 0.01%	0.18 ± 0.14%	3.1 ± 1.2%
3 h	28.6 ± 5.5%	24.8 ± 4.4%	6.8 ± 0.1%	0.11 ± 0.14%	0.47 ± 0.21%	4.4 ± 1.1%
6 h	40.2 ± 4.0%	30.2 ± 3.3%	8.1 ± 3.4%	0.04 ± 0.05%	0.09 ± 0.03%	4.1 ± 0.9%
24 h	50.0 ± 4.4%	34.0 ± 1.0%	10.0 ± 3.3%	0.08 ± 0.07%	0.18 ± 0.01%	7.9 ± 3.9%

Mean ± SD amounts of ^{166}Ho, Ho and acetylacetonate in the supernatant after resuspension and two times centrifuging of [^{166}Ho]Ho-AcAc-PLLA microspheres in the resupension media containing 2% Pluronic® F-68, and 10% ethanol or 116 mM phosphate pH 7.2 buffer after relative long neutron irradiation of 8.9×10^{20} thermal neutrons m^{-2}. AcAc: acetylacetonate only, no radiolytic products.

560 mg/kg for non-radioactive holmium was reported in rodents [28].

Different ^{166}Ho and Ho amounts in the blood and urine samples suggested no equilibrium and a different biological behaviour of their chemical forms (Tables 2–3). To study this in more detail, in vitro analyses were made after near-maximum neutron irradiation conditions were executed (Table 4). The higher amount of ^{166}Ho compared to Ho in the 10% ethanol buffer could be the result of the recoil of ^{166}Ho and the release from the acetylacetonate complex [25–27]. In addition, HPLC showed acetylacetonate release (Table 4). The lower detected amount of acetylacetonate in vitro compared to Ho and ^{166}Ho is most likely due to radiolysis, which is extensively described in the literature [29]. At least one other unknown irradiation-induced compound was detected which could be methyl acetonate, monomeric lactate or lactide, which may explain the yellowish discolouration. Similar results were found for neutron irradiated [^{166}Ho]Ho-AcAc microspheres which showed disintegration and high [^{166m}Ho]Ho^{3+} releases after incubation in water for injection [30].

In hindsight, the use of the 10% ethanol and 2% Pluronic® F-68 solution was suboptimal. The reason for using this solution was the tendency to aggregation of the hydrophobic [^{166}Ho]Ho-AcAc-PLLA microspheres after neutron irradiation, which could lead to obstructions in the catheter or an undesirable biodistribution. The 10% ethanol and 2% Pluronic® F-68 solution was implemented to improve the solubility and wettability of the [^{166}Ho]Ho-AcAc-PLLA microspheres just before the start of the clinical studies, but after the previous stability studies [31]. The earlier reported stability of [^{166}Ho]Ho-AcAc-PLLA microspheres was studied in vitro using phosphate buffered saline. This probably influenced the interpretation of the results, as discrimination between [^{166}Ho]$HoPO_4$ precipitate and [^{166}Ho]Ho-AcAc-PLLA microspheres was not shown [21,32,33]. Indeed, holmium precipitates after use of the phosphate-containing resuspension medium (Table 4). However, separation of the [^{166}Ho]$HoPO_4$ precipitates from [^{166}Ho]Ho-AcAc-PLLA microspheres resuspended in 116 mM phosphate buffered saline could only be distinguished indirectly using size distribution analysis and light microscopy.

Ho^{3+} can also form precipitates/nanocolloids with blood components such as phosphate and albumin. A first pass effect/phagocytosis by the reticuloendothelial system (RES) in the liver could be an explanation for the limited detection of holmium in blood and urine and for high, but varying liver detection and distribution on ^{166}Ho-SPECT-CT and Ho-MRI scans, especially after long irradiation times [14]. Other speculations for retention in the liver in such cases are decreased blood flow after embolization and high local concentrations of phosphates in the tumour regions due to necrosis [34].

Pharmacokinetic analyses in this study were limited by the number and timing of the blood samples and cumulative urine collection. This was done for pragmatic reasons such as multiple administrations and logistics like transport from the intervention room to the ward. Earlier sampling, for example, at five, 15 and 30 min, as performed in chemoembolization, would be of interest especially, because a fast initial release is suspected [35]. Furthermore, the cancer type and burden of disease could be of influence as it is a known variable in the hepatopulmonary shunt fraction. For example, the [^{99m}Tc]Tc-macroaggregates hepatopulmonary shunt fraction in metastases from colorectal cancer and hepatocellular cancers appears to be higher as compared to breast cancer. Also patients with compression or tumour thrombosis of a major portal vein branch have higher degrees of shunting than patients with normal portal vein perfusion [36]. The tumour to liver parenchyma ratio could also influence their distribution to the blood.

The quality of the product [^{166}Ho]Ho-AcAc-PLLA microspheres can be improved by minimization of the neutron irradiation damage. The use of additional gamma ray shielding during irradiation or a different neutron irradiation source could be used to decrease the damage, although the sterilization purpose should be kept in mind as well [37]. In addition, other matrices especially inorganic ones, whether or not they are combined with afterloading, could be considered to ease product characterization and to increase the time for transportation and logistics [18,30,38–42]. Finally, the product characteristics were somewhat different for each patient treatment in the HEPAR 1 and 2 studies. Therefore, a constant number of 6.6×10^{20} neutrons m^{-2} thermal neutrons and fixed logistics were chosen for new patient studies.

In conclusion, holmium amounts in peripheral blood and urine samples were low in all patients after intra-arterial injection of [^{166}Ho]Ho-AcAc-PLLA microspheres. A further decline of holmium amounts in blood and urine samples was detected after the reformulation of the resuspension solution, but neutron irradiation induced production damage should be minimized.

Conflict of interest

M.G.E.H. Lam is trainer and speaker on Quirem/Terumo sponsored events and consultant for BTG.

B.A. Zonnenberg holds patents related to holmium microspheres, which are assigned to University Medical Center Utrecht Holding BV. He receives honoraria from GSK Netherlands and consulted for, received research funding from and had expenses reimbursed by Novartis pharm Inc.

The department of Radiology and Nuclear Medicine of the UMC Utrecht receives royalties and research funding from Quirem Medical B.V. and Terumo.

Acknowledgements

The authors would like to acknowledge Dr. J.F.W. Nijsen and Dr. A.D. van het Schip for their contributions in product management and study designs, and G. van Bemmel and B. Aolad-si Mhammad for their help with analyses and data management.

R.C. Bakker is funded by the Dutch Cancer Society research grant: 2014-7075.

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