Are radiopharmaceuticals self-sterilizing? Radiation effect of gallium-68 and lutetium-177 on bacillus pumilus and staphylococcus succinus

Sind Radiopharmazeutika selbst-sterilisierend? Der Effekt von Gallium-68 und Lutetium-177 auf das Wachstum von Bacillus pumilus und Staphylococcus succinus

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ABSTRACT
Background For radiopharmaceuticals, aseptic preparation in combination with filtration is the most commonly used sterilizing method. In general, the production of radiopharmaceuticals needs to fulfill the requirements of good manufacturing practice. In the scope of this work, we focused on the positron emitter gallium-68 and on the therapeutically used beta- and gamma-emitter lutetium-177, as they are routinely used for in-house synthesis of radiopharmaceuticals in nuclear medicine departments. Our hypothesis is, that radiopharmaceuticals might be self-sterilizing due to a high radioactivity concentration and high-energy radionuclides in the preparation for intravenous injections.

Results Incubation with gallium-68 and lutetium-177 for both 30 minutes and 5 hours post-dispensing did not cause any significant effect on bacteria growth. As the theoretical dose is only 0.1–0.6 % of the Ph. Eur. recommended dose of 25 kGy, we conclude that the beta and positron energy of lutetium-177 and gallium-68 as used for standard radiopharmaceutical in-house production is not sufficient to decrease the number of colony forming units compared to the control values.

Conclusions Based on these findings, gallium-68 and lutetium-177 labeled radiopharmaceuticals are not self-sterilizing under the tested conditions with respect to bacillus pumilus and staphylococcus succinus. Consequently, strict aseptic preparation conditions in addition to end-sterilization of the radiopharmaceutical e. g. through membrane filtration are strongly advised for in-house productions.

ZUSAMMENFASSUNG
Hintergrund Für Radiopharmazeutika ist die aseptische Herstellung in Kombination mit der Filtration die am häufigsten verwendete Sterilisationsmethode. Generell müssen bei der Herstellung von Radiopharmazeutika die Anforderungen der guten Herstellungspraxis erfüllt werden. Im Rahmen dieser Arbeit haben wir uns auf den Positronenstrahler Gallium-68 und auf den therapeutisch genutzten Beta- und Gamma-Strahler Lutetium-177 konzentriert, da diese routinemäßig für die Inhouse-Produktion von Radiopharmaka in nuklearmedizinischen Abteilungen verwendet werden. Unsere Hypothese ist, dass Radiopharmaka aufgrund einer hohen Radioaktivitätskonzentration und hochenergetischer Radionuklide in der Zubereitung für intravenöse Injektionen selbststerilisierend sein könnten.

Ergebnisse Eine Inkubation mit Gallium-68 und Lutetium-177 sowohl für 30 Minuten als auch für 5 Stunden nach der Zuberei-
Sterility assurance level (SAL) of $10^{-6}$

Poetzsch S et al. Are radiopharmaceuticals self-sterilizing?

In the European Pharmacopoeia, six different methods are described to achieve sterility with a sterility assurance level (SAL) of $10^{-6}$ – steam sterilization, dry heat, radiation, gas, membrane filtration and working under aseptic conditions (Ph. Eur. Vol 9.0, 5.1.1). For radiopharmaceuticals, aseptic preparation in combination with membrane filtration is the most commonly chosen sterilizing method. Other methods are usually not applicable due to the short half-lives of the radionuclides and/or incompatibility of the radiopharmaceutical with elevated temperatures as they may contain heat-sensitive biomolecules. In general, the production of radiopharmaceuticals needs to fulfill the requirements of good manufacturing practice (GMP) and sterile (starting) materials are used wherever possible. The (automated) production is performed under aseptic conditions in class A laminar-air flow hoods or in hot cells/isolators using (automated) production is performed under aseptic conditions in class A laminar-air flow hoods or in hot cells/isolators using synthesis modules in GMP clean rooms. The final step of the production is usually the filtration of the radiopharmaceutical through a 0.22 µm membrane filter for end-sterilization. In order to check for sterility conformity, the radiopharmaceutical is incubated (retrospectively) for two weeks on growth media as described in the European Pharmacopoeia (Ph. Eur. Vol 9.0, 2.6.1).

Our hypothesis is, that radiopharmaceuticals might be self-sterilizing due to the fact that they contain a high radioactivity concentration and high-energy radionuclides. To the best of our knowledge, this hypothesis was thus far only tested for $[^{99m}Tc]$-radiopharmaceuticals [2, 3] and $[^{18}F]$-radiopharmaceuticals [4]. Brown et al. state, that the time lag between preparation and the sterility test of $[^{99m}Tc]$-radiopharmaceuticals should be as short as possible. Their reasoning is, that longer time lags have a greater chance to obtain negative sterility results due to a higher radioactivity dose, although the preparation might have been already contaminated at the time of dispensing. Jörg et al. investigated the “autosterilization” effect of $[^{18}F]$-radiopharmaceuticals and concluded, that intrinsic $[^{18}F]$-radiation is not sufficient for achieving sterility of the radiopharmaceutical. In our opinion, the main drawbacks, which we will avoid in our experiment set-up of these studies, were the long incubation times of up to 11 hours with the radiation source and the direct inoculation of the radiopharmaceutical of interest with the microorganisms. These long incubation times are not realistic in the daily routine because in-house synthesized radiopharmaceuticals in nuclear medicine departments are usually administered to the patients within one hour after preparation. Furthermore, samples taken directly out of the inoculated microorganisms-radiopharmaceutical solution contain remaining radioactivity. Thus, the seeded sample on growth media may be effected by the remaining radioactivity.

In the scope of this work, we will focus on the positron emitter gallium-68 and on the therapeutically used beta- and gamma-emitter lutetium-177, as they are routinely used for in-house synthesis of radiopharmaceuticals in nuclear medicine departments. Lutetium-177 is primarily a beta-emitter (490 keV) that decays after 6.7 days to the stable hafnium-177 but also emits gamma rays with an energy of 113 keV (3 %) and 210 keV (11 %). Gallium-68 is a positron emitter (1899 keV (88 %) and 822 keV (1 %)) with gamma energies of 511 keV (178 %) and 1077 keV (3 %) with a half-life of 68 minutes [5]. Both radionuclides can be linked to peptides such as DOTATOC (edotreotide) and PSMA [6].

**Results**

**Staphylococcus succinus**

The post-dispensing results of the 30 minutes and 5 hours incubations are shown in Fig. 1. After 30 minutes incubation, the number of colony forming units (cfu) in the control fraction increases compared to the sterility test of $[^{99m}Tc]$-radiopharmaceuticals should be as short as possible. Their reasoning is, that longer time lags have a greater chance to obtain negative sterility results due to a higher radioactivity dose, although the preparation might have been already contaminated at the time of dispensing. Jörg et al. investigated the “autosterilization” effect of $[^{18}F]$-radiopharmaceuticals and concluded, that intrinsic $[^{18}F]$-radiation is not sufficient for achieving sterility of the radiopharmaceutical. In our opinion, the main drawbacks, which we will avoid in our experiment set-up of these studies, were the long incubation times of up to 11 hours with the radiation source and the direct inoculation of the radiopharmaceutical of interest with the microorganisms. These long incubation times are not realistic in the daily routine because in-house synthesized radiopharmaceuticals in nuclear medicine departments are usually administered to the patients within one hour after preparation. Furthermore, samples taken directly out of the inoculated microorganisms-radiopharmaceutical solution contain remaining radioactivity. Thus, the seeded sample on growth media may be effected by the remaining radioactivity.

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**Results**

**Staphylococcus succinus**

The post-dispensing results of the 30 minutes and 5 hours incubations are shown in Fig. 1. After 30 minutes incubation, the number of colony forming units (cfu) in the control fraction increases to approximately double the initial number. The gallium-68 and lutetium-177 treated samples show a three-fold increase of cfu after 30 minutes of incubation at room temperature in 0.9 % NaCl solution. The increases in cfu are not significant compared to control values ($p = 0.44$ for gallium-68 and $p = 0.42$ for lutetium-177).

After 5 hours of incubation, the control and radiation treated samples show no significant change in cfu ($p = 0.45$ for gallium-68 and $p = 0.77$ for lutetium-177).
Fig. 2 shows the pre-dispensing results. After 30 minutes of incubation with 10% ethanol, the cfu decrease to approximately 27% (p = 0.02). After 15 minutes at 95 °C, there is no bacteria growth (p = 0.003).

**Bacillus pumilus**

The results of the 30 minutes and 5 hours incubation experiment post-dispensing are shown in Fig. 3. After 30 minutes, the amount of cfu in the control fraction does not change compared to the start value. The gallium-68 and lutetium-177 treated samples show no significant change compared to the control values (p = 0.30 for gallium-68 and p = 0.83 for lutetium-177) of cfu after 30 minutes of incubation at room temperature in 0.9% NaCl solution.

After 5 hours of incubation, the gallium-68 treated samples show a trend towards a decrease in growth (p = 0.17), although without statistical significance. The lutetium-177 treated samples show no significant change in growth compared to the control value (p = 0.89).

Fig. 2 shows the results of the pre-dispensing experiments. After 30 minutes of incubation with 10% ethanol, the cfu decrease to approximately 83% (p = 0.05). After 15 minutes at 95 °C, no bacteria growth was observed (p = 0.001).

**Theoretical dose**

The theoretical radiation dose was calculated using standard radiochemical formulas (Supporting Information). The theoretically achievable dose in the radiopharmaceuticals produced in-house ranges from 45 Gy to 161 Gy for gallium-68 and 12 Gy to 119 Gy for lutetium-177. Compared to the radiation dose to achieve an SAL of 10^-6, we achieve only 0.1–0.6% of the necessary dose of 25 kGy (Table 1).

**Discussion**

The growth behavior of two different microorganisms have been evaluated at two different steps during the preparation – either pre-dispensing or post-dispensing. **Bacillus pumilus** is a known radiation resistant species and can be used to validate ionizing radiation sterilization as used in the European Pharmacopoeia as sterility marker [7]. Additionally, this microorganism is resistant to environmental stresses. **Staphylococcus succinus** was chosen as a member of the wide-spread genus staphylococcus [8]. Both microorganisms are Gram-positive and categorized in the lowest biological safety class and can be handled in normal laboratories.

As gallium-68 and lutetium-177 labeled radiopharmaceuticals are mostly prepared in-house, we assumed that the time of injection will be within 30 minutes post-dispensing. Consequently, we chose 30 minutes as first incubation time point. We also added a 5
hours incubation point post-dispensing, as this might be relevant for lutetium-177 radiopharmaceuticals when they are produced ahead of injection.

Additionally, pre-dispensing measures were chosen. During the synthesis of e.g. [68Ga]Ga-DOTATOC, the starting materials are heated to 95 °C for approximately 15 min. Here, we tested the effect of an elevated temperature on the bacteria after 15 minutes. Additionally, gallium-68 radiopharmaceuticals are often post-processed with a maximum of 10 % (V/V) ethanol. To take this also into account, we tested the bacterial growth after 30 min incubation with 10 % (V/V) ethanol.

Within 30 minutes and 5 hours of incubation, the gallium-68 and lutetium-177 treated samples show no significant decrease of colony forming units. As the theoretical dose is only 0.1–0.6 % of the Ph. Eur. recommended dose of 25 kGy, we conclude that the beta and positron energy of lutetium-177 and gallium-68 are not sufficient to decrease the number of cfu compared to the control value. Sterility can thus not be achieved through self-sterilization by the chosen radionuclides.

Conclusions
Based on these findings, gallium-68 and lutetium-177 labeled radiopharmaceuticals are not self-sterilizing under the tested in-house production conditions with respect to bacillus pumilus and staphylococcus succinus. Consequently, strict aseptic preparation conditions in addition to end-sterilization of the radiopharmaceutical e.g. through membrane filtration are strongly advised for in-house productions.

Material and methods
All materials used were sterile single use materials e.g. syringes. Gallium-68 was eluted from the pharmaceutical grade 68Ge/68Ga generator (GalliaPharm, Eckert & Ziegler Radiopharma GmbH, Germany). Lutetium-177 was obtained from ITG Garching. Bacillus pumilus (DSM 492) and Staphylococcus succinus (DSM 105508) were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Radioactivity counting was performed using a borehole counter (Nuklear-Medizintechnik Dresden GmbH, Germany). Cultivation media tryptone soya broth USP was obtained from OXOID Deutschland GmbH. Bacteria concentration was calculated with plate count agar (146269) bought from Merck KGaA. All other chemicals were obtained from Sigma Aldrich.

Cultivation of bacteria
Bacillus pumilus and staphylococcus succinus are stored at -80 °C in tryptone soya broth USP with 25 % (V/V) glycerol as cryoprotectant. For every experiment, an overnight culture was established from the frozen culture. For that, a small amount of frozen bacteria culture was transferred with a sterile pipette tip into a sterile 15 mL falcon tube filled with 14 mL NaCl solution. After homoge-
nizing the bacteria solution, 50 µL was transferred into 10 mL cultivation media for overnight culture at 37 °C.

Post-dispensing experiments

The overnight culture was homogenized. The bacteria vials for the control and gallium-68/lutetium-177 treatment were prepared as follows: One mL (approximately 1 × 10^9 cfu) of the overnight culture was added to 9 mL 0.9 % NaCl solution and homogenized. A sample from this solution was taken to estimate the cfu at the beginning of the experiment (start). As control values, samples were taken from the untreated bacteria-solution after 30 minutes and 5 hours (control).

A 1.5 mL glas vial (1 mm thick) containing either 1 mL gallium-68 (approx. 1–2 GBq) or 1 mL lutetium-177 (approx. 0.8–1.4 GBq) was immersed into the 0.9 % NaCl bacteria vial for 30 min or 5 hours. Before taking samples at 30 min or 5 hours, the bacteria vial was homogenized.

100 µl of the bacteria vial was used for further dilutions and seeding on counting agar plates. After 24 h, the cfu were counted and the total cfu calculated as described below.

Pre-dispensing experiments

The overnight culture was homogenized. The bacteria vials for the ethanol and 95 °C treatment were prepared as follows: One mL (approximately 1 × 10^9 cfu) of the overnight culture was added to 9 mL 0.9 % NaCl solution and homogenized in case of the 95 °C treatment. For the ethanol treatment, 1 mL sterile ethanol was added to 8 mL 0.9 % NaCl solution and one mL of bacteria were added.

100 µl of the bacteria vial was used for further dilutions and seeding on counting agar plates. After 24 h, the cfu were counted and the total cfu calculated as described below.

Calculation of total cfu

For calculation of the total cfu, a dilution series of the bacteria vial was taken into account. (Table 2)

Overall the number of bacteria in the bacteria vial after the incubation time can be calculated by number of colonies * dilution * 100.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of Interest

The authors declare that they have no conflict of interest.

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