Evaluation of [^{67}Ga]Citrate in The Detection of Various Microorganism Infections in Animal Models

Original Article

Amir R. Jalilian[1], Aytak Novinrooz[2], Farahnaz Motamedi-Sedeh[1], Sedigheh Moradkhani[1], Amir A. Rajamand[1], Jalal Solati[2]

[1]Radiopharmaceutical Research and Development Laboratory, Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran, Tehran, Iran
[2]Karaj Islamic Azad University, Karaj, Iran

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ABSTRACT

Introduction: Gallium-67 citrate has been known as a good infection agent in nuclear medicine for decades. In this work the value of ^{67}Ga-citrate has been investigated in infected animal models using SPECT imaging at optimized/standardized conditions.
Methods: The bacterial (*Staphylococcus aureus; S.a. and Escherichia coli; E.c.*) and fungal (*Candidae albicans; C.a.*) species from standard sources were cultured according to the standard procedures and wild-type NMRI rats were inoculated by the injection of $5x10^7$ microorganisms (MO) into their thighs and animals incubated for infection site formation for 2 and 3 days followed by iv injection of freshly prepared ^{67}Ga-citrate (45-50 µCi) and SPECT imaging performed at 2 ,4 and 24 hours post injection in parallel with control groups.
Results: In *S.a.*-infected rats ^{67}Ga-citrate demonstrated hot spot foci at all time intervals *esp.* 24h post injections in contrast with normal animal scans. In case of *C.a.*, the infected animals also demonstrated significant accumulation foci being most significant after 24h. In *E.c.*-infected animals however weak positive scans were obtained even after 24 hours.
Conclusion: Our animal models developed for the evaluation of new infection-targeting agents were successfully positive using ^{67}Ga scan. These models can also be used in the evaluation of newly developed antibiotics in animal models for *in vivo* studies. The efficacy of ^{67}Ga-scan in our microorganism infection models can be summarized as *S.A.>C.a.>E.c.*.

Key words: Gallium-67 scintigraphy, Infection animal models, SPECT, Staphylococcus aureus, Escherichia coli, Candidae albicans

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Corresponding author: Dr Amir R. Jalilian, Radiopharmaceutical Research and Development Laboratory, Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran, North Kargar Ave. Tehran, Iran
E-mail: ajalilian@nrcam.org

INTRODUCTION

Over the past 30 years, a wide variety of radiopharmaceuticals have been proposed for the scintigraphic detection of inflammatory and infectious diseases. All radiopharmaceuticals yield a functional image of a process placed somewhere in the cascade of reactions in inflammation/infection (1, 2). Gallium-67 is a bioisoester of ferric iron with physical half-life of 3.3 days (78 hr) and biologic half-life of 2-3 weeks that binds to serum *via* transferrin, haptoglobin, albumin and globulins. Gallium attaches to tissues mediated by lactoferrin, as well as lymphocytes and macrophages. Ga-67 citrate remains a major tool for the detection of inflammation and infections (Figure 1).

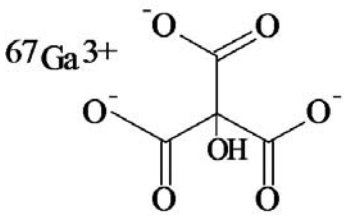

Figure 1. Structure of [^{67}Ga]-citrate

The interesting physical properties and availability of gallium-67 make it an interesting radionuclide for nuclear medicine diagnosis and research (3).^{67}Ga is medium half life (78 h) radionuclide with electron capture mode of decay (EC to ^{67}Zn), with 3, 185 and 300 keV gamma energies which is mainly produced using ^{68}Zn(p,2n)^{67}Ga reaction.

Gallium-67 citrate is absorbed to infection/inflammation zones and has been used in many diseases processes such as fever of unknown origin (FUO), severe lymphocytic inflammation (4) and autoimmune-based inflammations or chronic pancreatitis (5). Table 1 summarizes some important usages of this radioisotope in inflammation (5).

Detection of fungal infections is also reported using gallium-67 citrate. For instance, ^{67}Ga scintigraphy has successfully showed high uptake in the muscle abscesses formed by *Aspergillus fumigatus* in human (10), *cryptococos neoformans*–induced pneumonia in HIV patients (11), *Alcaligenes fecalis*-derived pericarditis in human (12), pulmonary aspergillosis in rodent models (13, 14), *Scedosporium apiospermum* infection in immunocompromsed patients (15) and also detection of sternal osteomyelitis due to *Candida krusei* and *Candida albicans* (16). There are also repetitive reports on the application of this tracer in bacterial infections (17), such as TB patients (18) and endocarditis patients (19).

There are however not many reports on the value of ^{67}Ga to differentiate various infections such as gram positive/gram negative and fungal infections. In this study, an experimental animal study has been proposed for the evaluation of Ga-67 citrate in the differentiation of these infections at the standardized/optimized setting followed by the presentation of valid infection animal models for future in vivo antibiotic therapy studies and/or infection imaging studies.

Table 1. Important applications of gallium-67 citrate in inflammation.

Application	Tissues	Reference
Idiopathic pulmonary fibrosis	Lung	(2)
Differentiation of malignant/benign/inflammatory	Oral/maxillofacial region	(6)
Pulmonary Wegener's granulomatosis	Chest/nasal region	(7)
Chronic bronchial asthma	Respiratory tract	(8)
Sarcoidosis	Pulmonary activity	(9)

METHODS

Enriched zinc-68 chloride with a purity of more than 95% was produced at Agricultural, Medical and Industrial Research School (AMIRS). ^{67}Ga was was also produced at the Nuclear Medicine Research Group at AMIRS 30 MeV cyclotron (Cyclone-30, IBA). Other chemicals were purchased from the Aldrich Chemical Co. (Gemany); and the ion-exchange resins from Bio-Rad Laboratories (Canada). Instant thin layer chromatography (ITLC) was performed by counting Whatman No. 2 papers using a thin layer chromatography scanner, Bioscan AR2000, Bioscan Europe Ltd. (France). Radionuclidic purity was checked with an HPGe detector, Canberra, U.S.A.. For activity measurement of the samples a recently calibrated CRC Capintech Radiometer (NJ, USA) was used. All calculations and ITLC counting were based on the 184 keV peak. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. The institutional and international guide for the care and use of laboratory animals was followed.

Production of ^{67}Ga-citrate

^{68}Zn(p,2n)^{67}Ga was used as the best nuclear reaction for the production of ^{67}Ga. Other impurities could be removed in the radiochemical separation process. After the target bombardment process, chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 M HCl (15 ml) and the solution was passed through a cation exchange resin (AG 50W, H+ form, mesh 200-400, h:10 cm, Ø:1.3 cm) which had been preconditioned by passing 25 ml of 9 M HCl. The column was then washed by 25 ml of 9M HCl at a rate of 1 ml/min to remove copper and zinc ions. To the eluent 30 ml water plus about 100 ml of a 6 M HCl solution was added. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100-200 mesh, h: 25 cm, Ø:1.7 cm) pretreated with 6 M HCl (100 ml). Finally, the gallium-67 was eluted as [^{67}Ga]$GaCl_3$ using 2 M HCl (50 ml); the whole process took about 60 min. To the final solution (25 ml, 250-50 mCi) was added 100 mg of sodium citrate and the mixture was stirred in a buffering flask for 10 minutes followed by filtration through a 0.22 micron membrane.

Quality control of the product

Control of Radionuclide purity: Gamma spectroscopy of the final sample was carried out counting in an HPGe detector coupled to a Canberra™ multi-channel analyzer for 1000 seconds.

Chemical purity control: This step was carried out to ensure that the amounts of zinc and copper ions resulting from the target material and backing in the final product are acceptable regarding internationally accepted standards. Chemical purity was checked by differential-pulsed anodic stripping polarography. The detection limit of our system was 0.1 ppm for both zinc and copper ions.

Induction of infections in wild-type rats

Microorganisms with specific ATCC numbers were purchased from Razi Institute, Karaj, Iran. The microorganisms were cultured in specific media at optimized conditions. Briefly, *Staphylococos aureos* was cultured in T.S.B. (Tryptic Soy Broth) medium for 24h at 37ºC. For *Escerichia coli* L.B. (Lactose Broth) medium was used for 24 hours at 37ºC and finally for *Candidiae albicans*, S.D.B. (saboroud's dextrose broth) medium was used for 48 h at 35ºC. The mixtures were homogenized and centrifuged at 2500 rpm and the cell pellets were reconstituted in physiological serum to

obtain a 10^7 M.O./ml (for bacteria) and 10^5 mo/ml (for fungi) observed by a 45X microscope neobar. To each wild-type rat weighing 180-220 g a mixture of the above yeast containing solution (0.1 ml each) was injected deeply in their thigh muscles under aseptic conditions (n=9).

Imaging of [^{67}Ga]-citrate in infected rats

After inoculation of rats with the various microorganisms (2 days for S.a. and E.c. and 7 days for C.a.) and waiting for optimized time, freshly prepared Ga-citrate (45-50 µCi, 50-100 µl) was injected through the rat tail veins under aseptic conditions. Images were taken 2, 4 and 24 hours after administration of the tracer by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. The useful field of view (UFOV) was 540 mm×400 mm. The spatial resolution was 10 mm FWHM at the CFOV. Sixty four projections were acquired for 30 seconds per view with a 64×64 matrix.

RESULTS AND DISCUSSION

Production

Gallium-67, in form of $GaCl_3$, was prepared by 24 MeV proton bombardment of the ^{68}Zn target at Cyclone-30 on a regular basis.

The target was bombarded with a current intensity of 170 µA and a charge of 1400µAh. The chemical separation process was based on a no-carrier-added method. Radiochemical separation was performed by a two-step ion exchange chromatography method with a yield of higher than 95%. Quality control of the product was performed in two steps. Radionuclidic control showed the presence of 93(40%), 184(24%), 296(22%), 378(7%) keV gamma energies, all originating from ^{67}Ga and showed a radionuclidic purity higher than 99% (E.O.S.).

The concentrations of zinc (from target material) and copper (from target support) were determined using polarography and shown to be below the internationally accepted levels, i.e. 0.1 ppm for Zn and Cu.

Radiolabeling

Because of the engagement of several polar functional groups in its structure, labeling of citrate with gallium cation affects its chromatographic properties and the final complex is more lipophilic. The free gallium remains at the origin of the paper as a single peak. In case of radiolabeling the more lipophil complex was tried using various conventional mobile pahses and many were able to distinguish $^{67}Ga^{3+}$ from the radiolabled complex. However, the suitable solvent used was methanol (Figure 2).

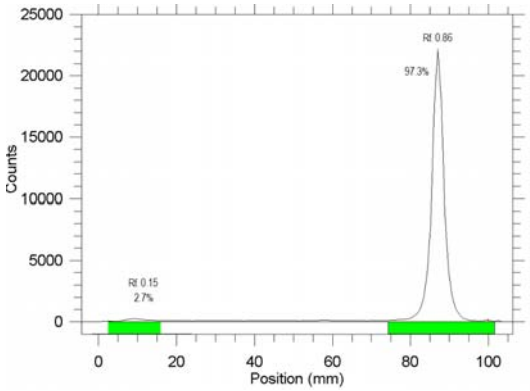

Figure 2. ITLC of [^{67}Ga]-citrate in pure methanol as mobile phase on Whatman No.2 papers.

Imaging of S.a.-infected rats using [^{67}Ga]-citrate

Imaging in the *S.a.*-infected rats showed a distinct accumulation of the radiotracer in the infected site around the thigh region (Figure 3) 2 h post injection where a visible lump can be diagnosed. The retention of the radioactive material in the target site was further investigated by anatomical study of the infection position after imaging and the M.O. was successfully cultured from the site. In figure 4(1a) significantly demonstrated the accumulation of the tracer at the infection site while at the control studies (1b) no significant difference was observed among the two thighs.

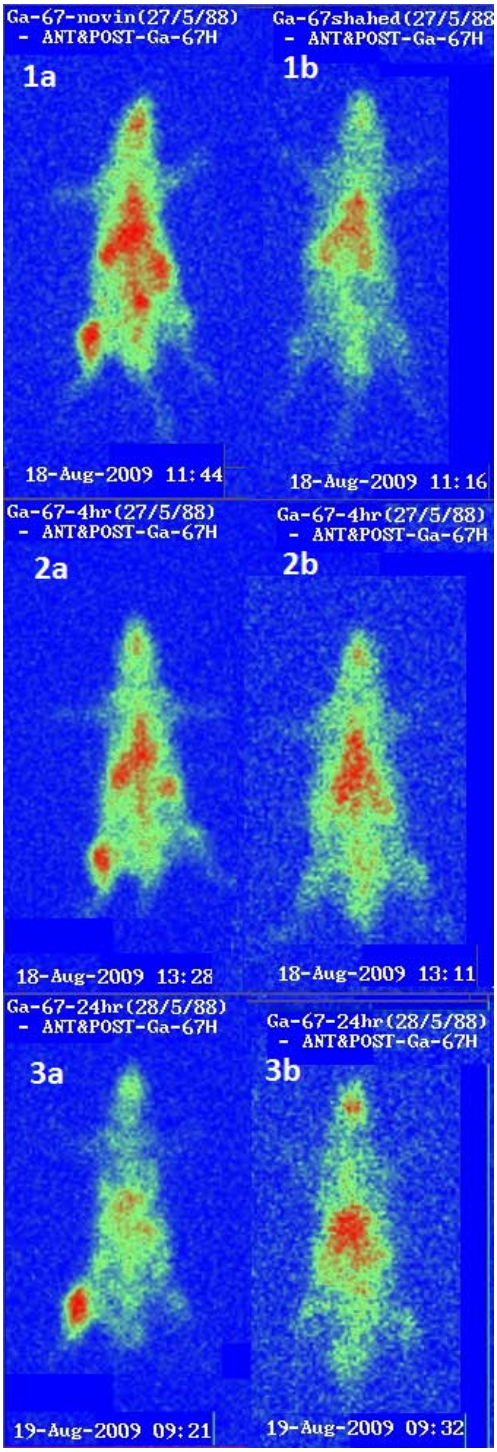

Figure 3. SPECT images of [^{67}Ga]-citrate (45-50 μCi) in *S.a.*-infected rats 2 (1a), 4 (2a) and 24 (3a) h post injection compared with negative controls at each time interval (1b-3b).

The same observation was made after 4h, while after 24 hours very significant infection site uptake is observed *esp.* due to the background reduction. Thus, Gramm-positive, *S.a.*, forms very distinguished infection site at our optimized conditions and chemotaxis leading to the attraction of the ^{67}Ga-tagged leucocytes is well performed due to nonspecific immune response.

Imaging of C.a.-infected rats using [^{67}Ga]-citrate

Candidae albicans has been known as a major cause of morbidity in HIV and immuno-compromised patients in last decades, thus we chose this MO as the fungal species model in our infection studies. Even 2 hours post injection of the tracer a slight positive uptake (1a) is observed in contrast with the control scans (1b) in Figure 4. This uptake increased 24 h so that a major difference among the test and the control groups was observed (3a). It can be suggested that the immune response in case of a fungal infection such as C.a. is not as rapid as Gramm-positive infection such as *S.a.* as shown in our repeated experiments (n=3).

Imaging of E.c.-infected rats using [^{67}Ga]-citrate

In Gramm-negative infected rats in our experiments no significant accumulation took place at least at 2h post injection and only a slight positive scan observed at 24h post injection (3a) (Figure **5**). Thus, in G$^-$-studies such as *E.c.*, ^{67}Ga imaging might not be the best option. On the other hand this might be due to the wrong choice of our animal model. In other similar studies, the animals were first immuno-compromised using chemotherapeutic agents for better formation of the infection sites, but we were trying to develop a positive scan in the normal animals with intact immune system.

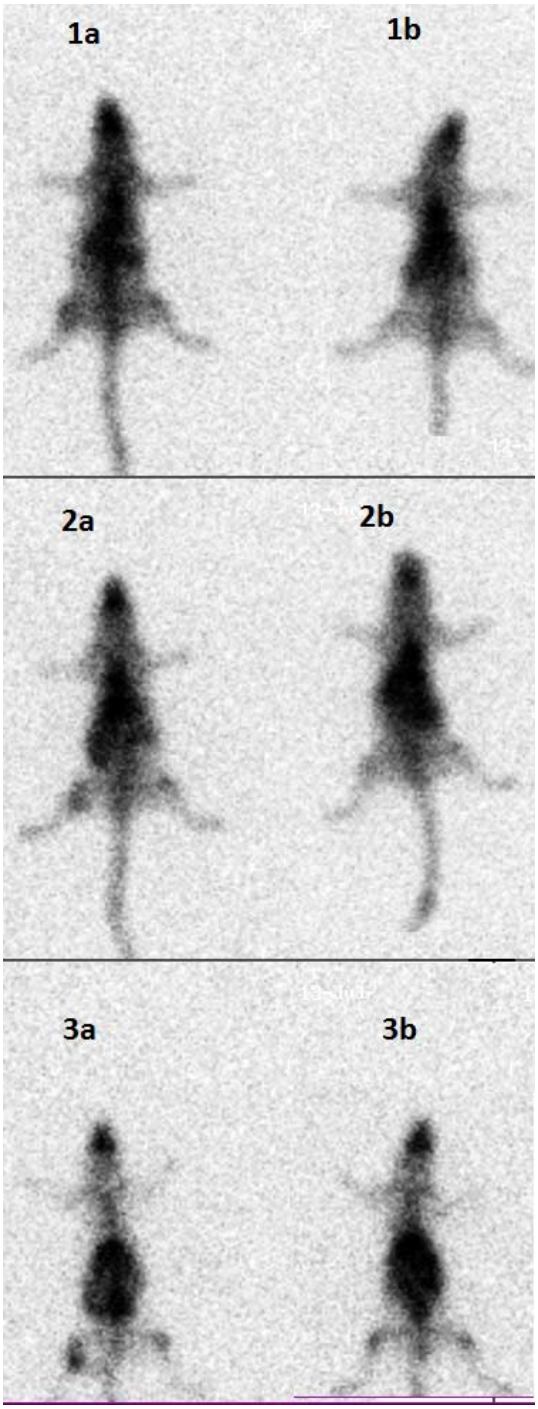

Figure 4. SPECT images of [^{67}Ga]-citrate (45-50 μCi) in *C. albicans*-infected rats 2 (1a), 4 (2a) and 24 (3a)h post injection compared with negative controls at each time interval (1b-3b)

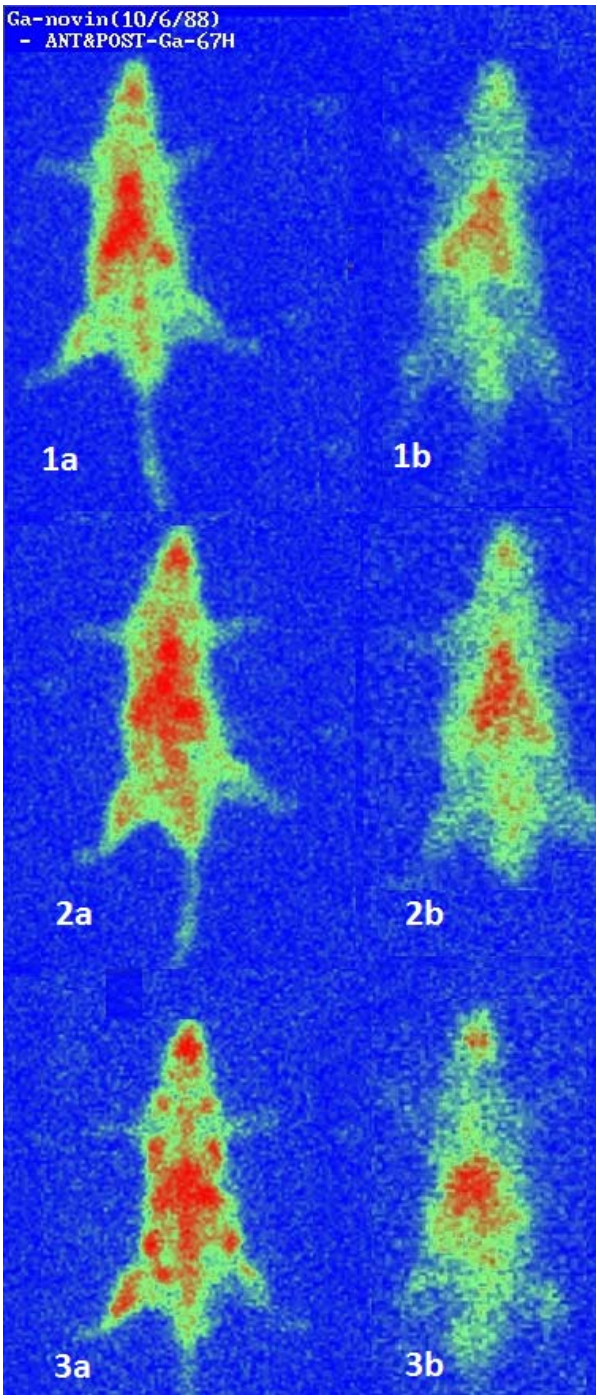

Figure 5. SPECT images of [^{67}Ga]-citrate (90 MBq, 22 μCi) in *E. coli*-infected rats 2 (1a), 4 (2a) and 24 (3a) h post injection compared with negative controls at each time interval (1b-3b)

The Gram positive cell wall is characterized by the presence of a very thick peptidoglycan layer, which is responsible for the retention of the crystal violet dyes during the Gram staining procedure . Embedded in the Gram positive cell wall are polyalcohols called teichoic acids, some of which are lipid-linked to form lipoteichoic acids. Because lipoteichoic acids are covalently linked to lipids within the cytoplasmic membrane they are responsible for linking the peptidoglycan to the cytoplasmic membrane. Teichoic acids give the Gram positive cell wall an overall negative charge due to the presence of phosphodiester bonds between teichoic acid monomers. Thus the strong entrapment of cations such as Ga^{3+} can be explained by the negatively-charged cell wall of G^{+} species, and this can be a good explanation to the distinct and significant diagnosis of S.a. infection in this study. While E. coli, being a gram negative microorganism has the lowest affinity for the positively-charged cations such as Ga^{3+}.

On the other hand the glycan cell wall of most yeasts including C.a. is almost a neutral-charged environment which do not specifically entrap cations such as Ga^{3+}.

Although there might be other reasons for the selective attachment of Ga^{3+} cation to extra/intra cellular components of these microorganisms, however, the retention as well as the diagnostic characteristics of ^{67}Ga-citrate can at least parttly be explained by the chemical structure and ionic charges of their cell walls.

The available, inexpensive and widely used ^{67}Ga-citrate has been a major tool for the detection of infections in nuclear medicine, while the use of other tracers such as radiolabeled polyclonal IgG as well as radiolabeled chemotactic peptides has been shown to be limited to their nonspecific false-positive results in many cases. Thus the application of ^{67}Ga-citrate seems to continue in the detection of various microbial infections. Considering the results of this paper as well as others, would lead to better understanding the value and the diagnostic time-frame in various G^{+}/G^{-} and fungal infections.

CONCLUSION

Biodistribution of the [^{67}Ga]-citrate in *E.c.*-infected rats demonstrated minor infection site uptake at 24h post injection. For S.a.-infected animals, on the other hand ^{67}Ga demonstrated significant accumulation in infection foci even after 2 h post injection, while it was most significant after 24 hours. In *C.a.*, as a life threatening MO in immunocompromised and HIV-positive patients, also ^{67}Ga scan demonstrated significant site uptake after 24h which is good enough specially for future studies. In conclusion, our animal models have shown excellent potential for S.a. infection studies and good results for C.a. infections while for E.c.-infections our protocol did not demonstrate satisfactory results.

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