The cationic peptide 68Ga-NOTA-UBI-29-41 was synthesized and characterized. Biodistribution and PET/CT examinations were performed for evaluation of its biologic behavior. Differentiation of infection from sterile inflammation was investigated using microbiology methods at the sites of bacterial infections. Methods: Labeling of UBI-29-41 conjugated with NOTA with 68Ga was optimized at 20°C–100°C and pH 3.5–5.5. Radiochemical purity, stability up to 260 min, and binding to serum proteins were determined. In vitro binding to Staphylococcus aureus was evaluated from 9.14 × 107 to 1.17 × 1012 cfu/mL. Of 3 groups of Mus musculus Swiss male mice, the first was inoculated intramuscularly with 1.2 × 108 cfu of S. aureus to provoke infection, and the second, with 1.2 × 109 cfu of heat shock–treated S. aureus to generate sterile inflammation. The third mouse was not treated and served as a control. After 24 h, 68Ga-NOTA-UBI-29-41 was administrated intravenously, and biodistribution was performed at 30, 60, and 120 min. PET/CT dynamic studies (120 min) were acquired. Sinograms were reconstructed using 3D maximum-likelihood expectation maximization algorithm methods at the sites of bacterial infections. The neutral Ga(III) complex of NOTA has been shown to undergo acid-catalyzed dissociation only at low pH levels, which is unlikely in physiologic conditions. The 68Ga-NOTA complex has high thermodynamic and kinetic stability (13). The aim of this study was to optimize parameters such as pH, buffer systems, and time of incubation that affect the labeling of 68Ga-NOTA-UBI-29-41 and its quality control. In vitro studies were performed using the radiolabeled peptide with increasing amounts of S. aureus. In vivo studies included the evaluation of the tracer in mouse models developed to express infection or sterile inflammation (14).
MATERIALS AND METHODS

Synthesis of $^{68}$Ga-NOTA-UBI-29-41

The 29-41 amino acid fragment of ubiquicidin (UBI-29-41) conjugated with NOTA (NOTA-UBI-29-41, molecular weight, 1978.25; purity > 90%; pICHEM) was used for $^{68}$Ga labeling. $^{68}$Ga was obtained as GaCl$_3$ or, alternatively, from a generator (IGG100; Eckert & Ziegler or Isotope Technologies Garching) by fractionated elution with HCl (31.5% by acidimetry, Suprapure; Merck) of 0.1 or 0.05 M, respectively, to get the highest activity concentration. Activity levels in 1.2- to 2.5-mL fractions ranged from 200 to 650 MBq. For the optimization of labeling parameters, 5–60 nmol of NOTA-UBI-29-41 and approximately 30 MBq of $^{68}$Ga were used. pH was determined before incubation at 20°C. The in vitro binding of $^{68}$Ga-NOTA-UBI-29-41 to $S$. aureus was measured by counting the colony-forming units of the bacteria in the sample. The $S$. aureus treated group consisted of healthy mice.

Animal Studies. Studies were performed according to national and international guidelines for the use of animals for research. The Bioethics Committee of the Uruguayan Centre of Molecular Imaging approved this study.

Preparation of Animal Models. Three groups of $M$. musculus Swiss male mice, 25–30 g, aged 11–12 wk, were used. In the first group, infection was induced by inoculation of 0.1 mL of $S$. aureus (1.2 × 10$^9$ cfu/mL) in the left upper thigh muscle. In the second group, inflammation was induced by inoculation of 0.1 mL of heat shock-treated $S$. aureus (1.2 × 10$^9$ cfu/mL) in the same region. The third group consisted of healthy mice.

Ex Vivo Studies. Biodistribution studies were performed 24 h after inoculation. Four animals per group were injected with 2.6 ± 0.5 MBq, 4.2 ± 1.3 MBq, and 7.5 ± 1.9 MBq (50–150 μL) of $^{68}$Ga-NOTA-UBI-29-41 in the caudal tail vein and were sacrificed by cervical dislocation at 30, 60, and 120 min after injection. Another group was injected with the radiopharmaceutical 48 h after inoculation with $S$. aureus. In these mice, biodistribution was studied at 1 h ($n = 2$) and 2 h ($n = 1$) after inoculation. All organs, samples of blood, and the thigh muscles (left as target, right as control) were dissected, weighed, and assayed for radioactivity. Total urine volume was collected during the biodistribution period or removed from the bladder after sacrifice, and its activity was measured. The percentage injected dose per gram of tissue and injected dose in the whole organ were calculated, as well as the target-to-nontarget (T/N) ratio of muscles.

Image Acquisition. PET/CT imaging in mice was performed using a trimodality scanner (Triumph; Gamma Medica, Inc.) with lutetium yttrium oxyorthosilicate and lutetium gadolinium oxyorthosilicate scintillators (LabPET) (spatial resolution, 1.0 mm; axial field of view, 3.75 cm). Data were acquired in list mode in a 184 × 184 × 31 matrix with a pixel size of 0.25 × 0.25 × 1.175 mm and a coincidence window width of 22.22 ns. The animals were anesthetized with 2% isoflurane in an oxygen flow of 2 L/min, placed prone on the scanner bed, and intravenously injected with 50–150 μL of $^{68}$Ga-NOTA-UBI-29-41 (7.5 ± 1.9 MBq). Dynamic studies were performed for all groups during 120 min (4 frames × 30 min and 24 frames × 5 min). Sinograms were reconstructed using 3D maximum-likelihood expectation maximization.

Image Analysis. Semiqualitative analysis was done using PMOD software, version 3.4. PET studies were coregistered with the corresponding CT studies for anatomic localization. Volumes of interest were drawn on 2 selected thigh muscle regions (left as target, right as control or nontarget) to analyze their specific uptake. SUVs normalized for injected dose and body weight were calculated and averaged for each group.

Confirmation of Infection. The disinfection of infected muscle in aseptic conditions and its homogenization and culture were performed to confirm the presence of infection and to correlate the specific uptake of $^{68}$Ga-NOTA-UBI-29-41 with the colony-forming units of...
S. aureus per gram of muscle (3). The dissected target and nontarget muscles were homogenized (T-25 digital ULTRA-TURRAX; IKA-Werke GmbH & Co.) in 5.0 mL of phosphate-buffered sulfate, 0.015 M, pH 7.2, and diluted 1:10 from 10^{-1} to 10^{-4}. One milliliter of each dilution was plated on trypticase soy agar and incubated for 24–48 h. The number of colony-forming units was determined, and results were expressed as cfu/g of tissue. The same procedure was performed for the contralateral muscle of infected muscles and for inflamed muscles.

RESULTS

Synthesis and Radiolabeling of $^{68}$Ga-NOTA-UBI-29-41

Optimization of Labeling Parameters. For pH evaluation, we used the range recommended (3.5–4.5) in the bibliography (1,8, 12,13,15), in which the effect of pH in the labeling with $^{68}$Ga is thoroughly analyzed. We also tested several buffer pH settings and concentrations to achieve robust conditions for the labeling, reaching optimal conditions for pH in the recommended range and minimizing salt concentration. The effect of temperature is shown in Table 1. High yields were obtained for both temperatures; but at 90°C the reaction labeling proceeded faster, requiring only 5 min to reach more than 95%.

Radiochemical Purity. As to whether 96% ± 3% ($n = 16$) was in the optimized conditions—90°C for 5 min and pH 3.5–4.0—Figure 1 shows a typical HPLC profile. Retention times for labeled and unlabeled UBI-29-41 were 6.825 ± 0.024 min and 6.746 ± 0.029 min, respectively. $^{68}$Ga colloid determination by Sep-Pak C18 correlated well with the HPLC and ITLC-SG results. The amount of colloid was 1.1% ± 1.3%. Labeling yield evaluated for different peptide-to-activity ratios is shown in Figure 2. Maximum yield and radiochemical purity were reached for 30 nmol of NOTA-UBI-29-41 and 30 MBq incubating at 20°C for 10 min. Specific activity calculated from the HPLC ultraviolet calibration curve was 0.55 MBq/nmol.

Stability of $^{68}$Ga-NOTA-UBI-29-41. The stability of the peptide was confirmed up to 3 h, showing no decrease from the initial 97% value during this time.

Binding of $^{68}$Ga-NOTA-UBI-29-41 to Proteins. The binding of $^{68}$Ga-NOTA-UBI-29-41 ranged from 37% to 52% during the time studied (30–120 min), as shown in Table 2.

Biologic Evaluation

In Vitro Binding of $^{68}$Ga-NOTA-UBI-29-41 to S. Aureus. We found a logarithmic increase dependent on the number of bacteria and reaching 95% ± 11% for 5 × 10^6 cfu, with a maximum of 97% ± 2% for 1.17 × 10^8 cfu (Fig. 3). Binding to bacteria is evident even for low amounts of S. aureus (19.2% of the total activity for 9.14 × 10^6 cfu).

TABLE 1

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>% yield*</th>
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<tbody>
<tr>
<td>20</td>
<td>93.7 ± 5.7 ($n = 5$)</td>
</tr>
<tr>
<td>90</td>
<td>95.9 ± 4.4 ($n = 5$)</td>
</tr>
</tbody>
</table>

*Values represent average ± SD of $n = 5$.

Biodistribution of $^{68}$Ga-NOTA-UBI-29-41. High renal excretion and rapid blood clearance were observed in all groups (Fig. 4). In healthy mice, activity eliminated by urine reached 83.2% ± 7.3% injected dose in 2 h. Swelling, increased vascularization, and pus were observed in the infected thigh muscle, but no pus was seen in the inflamed muscles. Even large lymph nodes were observed in infected mice. Microbiologic assays determined that the changes were related to bacterial growth. The ratio of infected muscle (activity/gram) or inflamed muscle compared with the healthy leg (T/NT) reached 5.0 at 60 min and 4.1 at 120 min. The injection of S. aureus was done 48 h before the administration of $^{68}$Ga-NOTA-UBI-29-41, the T/NT ratio reached 4.16 ± 0.02 ($n = 2$) 1 h after injection and 9.4 ($n = 1$) at 2 h. ANOVA and Tukey statistical testing showed a $P$ value of 0.002 or less for the T/NT of the group of infected mice compared with the groups of inflamed and healthy mice for all the times studied. No significant differences ($P = 0.817$) were found among the groups of healthy and inflamed mice, demonstrating the ability to distinguish infection from inflammation.

PET/CT Imaging. T/NT ratios calculated from PET/CT images for infected mice were greater than 2.4 at 10 min, increased gradually to a maximum of more than 4.0 at 60 min, and decreased slightly after 80 min (T/NT > 3.4) after injection (Fig. 5B). All...
infection foci could be visualized on PET/CT (Fig. 6). The $^{68}$Ga-NOTA-UBI-29-41 uptake was significantly higher in infected than in inflamed mice.

**Confirmation of Infection.** Culture of homogenized muscles confirmed the presence or absence of *S. aureus* (Fig. 7). *S. aureus* was not found in any of the contralateral muscles, nor was it detected in any of the muscles of inflamed mice. Culture of the excised infected muscle revealed the presence of viable *S. aureus*, correlating with the T/NT values. Even with levels of $2.5 \times 10^2$ cfu/g, the T/NT was 2.5 at 60 min after injection, whereas for inflammation, the T/NT at the same time was 1.6.

**DISCUSSION**

In this study, $^{68}$Ga-NOTA-UBI-29-41 was labeled and characterized by HPLC, ITLC-SG, and SPE. High yield and radiochemical purity (>96%) were obtained with no need of further purification. The radiolabeled peptide was stable up to 3 h.

The analytic systems used to determine and quantify the different radiochemical impurities, including $^{68}$Ga colloid, were successful and in concordance with previous studies (16).

$^{68}$Ga is obtained from a $^{68}$Ge/$^{68}$Ga generator (15, 17–19), which facilitates the labeling of NOTA-UBI-29-41 for clinical use in sites having no cyclotron. Previously, we have used SPECT with $^{99m}$Tc-UBI-29-41 in infected mice and rabbits (4, 5, 20, 21). In this study, we have used $^{68}$Ga-NOTA-UBI-29-41 to analyze its in vivo biology in infection, inflammation, and healthy mouse models and to evaluate its potential as a PET/CT tracer for the targeting of bacterial infection. Our aim is to transfer previous knowledge to this new positron-emitter radionuclide without compromising the infection imaging properties of UBI-29-41. The labeling of this peptide with $^{99m}$Tc was done by a direct method of reduction with KBH$_4$ and SnCl$_2$ involving Arg and Lys residues (22). For the labeling with $^{68}$Ga otherwise, a chelating agent attached to Thr is required. DOTA is the most commonly used chelating agent, but we selected NOTA because it has a smaller diameter, greater stability, faster labeling kinetics, and greater in vivo stability (13). The NOTA-UBI-29-41 was labeled using $^{68}$Ga, which was eluted from an organic-based generator by fractionated elution, a simple but reliable method to increase concentration. Labeling was successful, and postpurification was not needed because of the high yields and the negligible amounts of colloid. Furthermore, we have confirmed that $^{68}$Ga-NOTA-UBI-29-41 is stable in vitro for at least 3 h. Biodistribution data of $^{68}$Ga-NOTA-UBI-29-41 in healthy mice at different times after injection revealed high urinary elimination, moderate uptake in the kidneys, and negligible uptake in the liver. This biologic pattern does not interfere with the image quality. Biodistribution studies indicated specific uptake in infected tissues as evidenced by the T/NT ratio of approximately 5 at 60 min after injection, maintaining values higher than 4.1 for up to 120 min. Studies in mice with $^{99m}$Tc-UBI-29-41 revealed a similar pattern (21). This indicates that the conjugation with NOTA and the further labeling with $^{68}$Ga do not alter the in vivo properties of UBI-29-41. Our study confirms values for percentage injected dose in liver, kidney, and bladder urine similar to those reported by Ebenhan et al. (23). All tissues except the kidneys showed a peak tracer concentration at 60 min that decreased slightly until 120 min. Our study also successfully determined the activity levels in blood and urine samples. We observed that 75% ± 14% of the injected activity was recovered in total urine 120 min after injection, which agrees with a previous study (21). Fifty-two percent of the $^{68}$Ga-NOTA UBI-29-41 was bound to plasma proteins in 2 h, which also agrees with the percentage distribution to blood proteins by time.

### TABLE 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% bound to plasma proteins*</th>
</tr>
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<tbody>
<tr>
<td>30</td>
<td>37.4 ± 0.2</td>
</tr>
<tr>
<td>60</td>
<td>44.1 ± 2.6</td>
</tr>
<tr>
<td>90</td>
<td>50.6 ± 2.6</td>
</tr>
<tr>
<td>120</td>
<td>51.7 ± 2.5</td>
</tr>
</tbody>
</table>

*Values are average ± SD of n = 2.

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**FIGURE 3.** Percentage of $^{68}$Ga-NOTA-UBI-29-41 bound to increasing amount of *S. aureus*, expressed as percentage of activity bound over total activity (% B/T) vs. colony-forming units ($n = 2$).

**FIGURE 4.** Biodistribution of $^{68}$Ga-NOTA-UBI-29-41 expressed as percentage of activity (%IA) per gram for each organ or tissue in healthy mice ($n = 4$) at 30, 60, and 120 min after injection (A) and in healthy, inflamed, and infected mice ($n = 4$) at 60 min after injection (B).
plasma fraction and blood cells (21). The two clinical studies performed with $^{99m}$Tc-UBI-29-41 support our findings of gradual blood activity decline and similar accumulation in urine (24,25).

It has been suggested that antimicrobial peptides bind selectively to bacterial cytoplasmic membranes. The cationic peptide residues are capable of interacting electrostatically with the anionic regions of bacterial membranes (14,26). As described by Ferro-Flores et al. (27), UBI-29-41 fragment is a cationic human antimicrobial peptide with 6 positively charged residues (5 Arg + 1 Lys) and no cysteine. In the direct labeling with $^{99m}$Tc, the amine groups of Lys and Arg 7 are presumably involved. Our labeling with $^{68}$Ga was made incorporating the radionuclide into the NOTA core attached to Thr; thus, neither Arg nor Lys is likely involved. This method enhances the overall cationic charge of the peptide molecule, increasing the probability of electrostatic interactions with the bacterial wall. Experimentally, this finding was reflected by the almost 100% reached for the in vitro binding of $^{68}$Ga-NOTA-UBI-29-41 to $S. aureus$.

Biodistribution and imaging studies were performed at 30, 60, and 120 min in mice bearing infection and sterile inflammation. The optimal time for T/NT ratio as well as for blood clearance was 60 min. $^{68}$Ga-NOTA-UBI-29-41 uptake was greater in infected muscle than in healthy tissue in 18 of 18 mice, indicating high sensitivity. We inoculated 3 mice with $S. aureus$ 48 h before the administration of $^{68}$Ga-NOTA-UBI-29-41 to confirm the degree of infection suitable for detection. We found similar results to those for the mice inoculated 24 h before the administration of the tracer. The muscular infection in mice produced significantly greater accumulation than in inflamed and healthy mice at all the measured times (T/NT, $P \leq 0.002$). No significant differences ($P = 0.817$) were found among the groups of healthy and inflamed mice (ANOVA and Tukey statistical test). T/NT ratios calculated from PET/CT images for infected mice allowed differentiation between infection and inflammation from 10 min (T/NT = 2.4) and increased gradually to a maximum of 60 min (T/NT > 4.0), decreasing slightly up to 120 min (T/NT > 3.4) after injection.

Culture of the excised infected muscle revealed the presence of viable $S. aureus$, correlating with the T/NT values (Fig. 7). Even amounts of bacteria on the order of $10^4$ were enough to discriminate infected from sterile inflamed or healthy mice 1 h after injection of $^{68}$Ga-NOTA-UBI-29-41, which clearly indicates that the radiotracer discriminates bacterial infection from sterile inflammation.

The in vitro binding of $^{68}$Ga-NOTA-UBI-29-41 to increasing amounts of $S. aureus$ showed that the binding capacity of UBI-29-41 was maintained despite the conjugation of NOTA to the peptide and the labeling with $^{68}$Ga. The maximum binding reached almost 100%, indicating the high specificity of the labeled molecule.

CONCLUSION

The labeling of $^{68}$Ga-NOTA-UBI-29-41 was optimized at different temperatures, times, and pH conditions. The optimal ratio activity per peptide was determined, allowing a high specific activity. In vitro binding of $^{68}$Ga-NOTA-UBI-29-41 to $S. aureus$...
shows that the labeling does not alter the binding capacity, which reaches a maximum of approximately 100% and indicates high sensitivity. High uptake of ⁶⁸Ga-NOTA-UBI-29-41 was observed by PET/CT and biodistribution studies in infected tissues. All the infection foci were clearly delineated by the PET/CT acquisitions from 30 min after injection. A positive correlation was found between the number of colony-forming units recovered from in-vivo. Compared with SPECT with ⁹⁹mTc-UBI-29-41, PET/CT with ⁶⁸Ga-NOTA-UBI-29-41 represents an important improvement. The uptake, distribution, and excretion of UBI-29-41 were not significantly compromised by the conjugation with NOTA despite the differences in structure from ⁹⁹mTc-UBI-29-41. Our findings demonstrate that ⁶⁸Ga-NOTA-UBI-29-41 can discriminate in vivo infection from inflammation, which means it has an excellent potential for clinical use. More research is necessary concerning the binding to other bacteria and fungi.

DISCLOSURE

The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734. No potential conflict of interest relevant to this article was reported.

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68Ga-NOTA-UBI-29-41 as a PET Tracer for Detection of Bacterial Infection

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