ORIGINAL PAPER

THE CHEMISORPTION-RELEASE AND ANTIBACTERIAL POTENTIAL STUDIES OF GENTAMICIN FROM HYDROXYAPATITE-BASED IMPLANTS

RENATA-MARIA VARUT*[1], VALENTIN MANDA[1], OANA GINGU[2], GABRIELA SIMA[2], CRISTINA TEISANU[2], JOHNY NEAMTU[1]

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Abstract. *The treatment of bone and joint infections is very difficult to achieve, the challenge being to create antibiotic-functionalized implants on their surface, for local release at the bone level of the pharmacologically active agent. In the present study we performed implants based on hydroxyapatite (HAp), HAp reinforced with titanium particles (HApTi) and HapTi with added calcium fructoborate (CaFb) by chemisorption deposition method (HApTiCaFb). The implants was immersed in gentamicin (GTN) solution for 48 hours, then was determined the release profile of antibiotic for 14 days and the antibacterial effect of the three types of composite. The period of antibiotic release may be considered as sufficient to support osteointegration under antibacterial protection.*

Keywords: *chemisorption, release, gentamicin, antibacterial effect*

1. INTRODUCTION

Treatment of bone and joint infections, such as osteomyelitis, septic arthritis, and joint prosthesis infections can be very difficult, requiring long-term administration of antibiotics and additional surgical treatment. The most common cause of acute or chronic hematogenic osteomyelitis in adults and children is *Staphylococcus aureus* infections. Group A *Streptococcus*, *Streptococcus pneumoniae* and *Kingella kingae* are pathogens that cause osteomyelitis in children. In chronic osteomyelitis, which may be caused by neighborhood infections, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Escherichia coli* were isolated. Osteomyelitis caused by fungal and mycobacterial infections is less common, being reported in patients with functional deficiencies [1].

In the present study aims to obtain HAp-based implants, which contain GTN adsorbed by the chemisorption method and we aimed to release the antibiotic for a period of 14 days. Also, the minimum inhibitory concentration (MIC) of GTN was established on the tested germs: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). Thereafter, the therapeutic efficacy of HAp-GTN, HApTi-GTN and HApTiCaFb-GTN was determined, compared to the MIC of the GTN (control +) over the standard reference bacterial strains. In the qualitative interpretation of the antibiograms, the aim was to respect the American standards, developed by CLSI (Clinical Laboratory Standards Institute).

[1] University of Medicine and Pharmacy Craiova, Faculty of Pharmacy, 200349 Craiova, Romania.
E-mail: rennata_maria@yahoo.com.
[2] University of Craiova, Faculty of Mechanics, 200512, Craiova, Romania.

Gentamicin is an aminoglycoside antibiotic used to treat several types of bacterial infections. This may include bone infections, endocarditis, pelvic inflammatory disease, meningitis, pneumonia, urinary tract infections, and sepsis among others [2]. It can be given intravenously, by injection into a muscle, or topically.

2. MATERIALS AND METHODS

2.1. DESCRIPTION OF IMPLANTS

The implanted biocomposite samples are made using the powder metallurgy technology. The matrix material is HAp ($Ca_5(PO_4)_3(OH)$) powder particles (Merck; average 200 nm particle size) which is reinforced by the titanium hydride (TiH_2) powder particles (Merck; ~100 μm; water atomized). The mixing content between the components is wt. 75% HAp and wt. 25% TiH_2. The first step of the biocomposite samples preparation consists in the HAp drying. One calcination cycle is developed in air at 900 °C for 1 hour. Then the calcinated matrix and reinforcement powder particles are mixed in a planetary ball mill for 30 minutes. The ratio between the milling balls (stainless steel, 5 mm diameter) and the powder mixture is 2:1. The milling environment is ethanol (1 mL / 1 g powder mixture), the obtained mixture is dried for 1 hour at 200 °C in a conventional furnace, followed by the extraction of the milling balls out of the powder mixture. The biocomposite samples are shaped using the cold compaction operation at 120 MPa in a metallic die of 10 mm as inner diameter on A009 electromechanical-computerized 100 kN testing machine, equipped with TCSoft 2004 Plus software. Then the compacts are submitted to the two steps sintering (TSS) heat treatment using a laboratory Nabertherm chamber furnace, type L5/12, max. temperature 1200 °C. A protective gas atmosphere (pure argon 99.98%) is provided along the entire treatment through the rear wall connection of the furnace [3]. During the first step of TSS the compacts heating reaches a peak temperature (T_1 = 900 °C) for a very short time (1 minute) to achieve an intermediate density by the initiation of the diffusion process between HAp and TiH_2 powder particles. Then the second step of TSS develops by means of a rapid cooling to T_2 = 800 °C and the dwell time is 10 hours. The final densification process occurs without the grain growth of the nanosized HAp particles [4-11]. To study the possibilities to improve the osseointegration of the biocomposite samples into the genuine bone, some sintered samples were immersed into a CaFb based solution 0.4 g/10 mL for 48 hours.

2.2. GTN CHEMISORPTION

All implants, weighed previously, were completely immersed for 48 hours in GTN solution (0.3 mg GTN/10 mL ultrapure water). Subsequently, the implants were deposited on filter paper, dried for 48 hours at room temperature, and then weighed.

2.3. RELEASE PROFILE OF GTN

Release studies were carried out on HAp-GTN, HApTi-GTN and HApTiCaFb-GTN implants, with ultrapure water being chosen as the release medium. The temperature was mantained in the oven at 37 °C ± 0.5 °C. A lid container was used to ensure tightness during the measurements. Thus, 5 mL of HPLC ultra-pure water (LiChrosolv, Merck) was initially added and then the test implants were immersed. At regular time intervals (1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 144 h, 216 h, 288 h and 336 h respectively) 0.5 mL solution was taken for quantitative determination of the GTN by the HPLC technique described. The volume taken was replaced with the release medium (ultrapure water) [12].

2.4. DETERMINATION OF THE GTN AMOUNT IN THE RELEASE MEDIUM BY HPLC

A Thermo Finnigan Diode Detector (DAD) HPLC system was used for this purpose; for the acquisition of the spectra a Thermo Finnigan Xcalibur data system was used. For the chromatographic analysis, a C18 Hypersil GOLD reverse phase column (250 mm x 4.6 mm I.D., particle size 5 μm) was used. The mobile phase was composed of acetonitrile/methanol/water (85/10/5, v / v/ v), isocratic elution with a flow rate of 1 mL / min. The determinations were made at 265 nm [13].

The calibration curve was obtained by dissolving the standard GTN in borate buffered solution at pH 9.7 in the concentration range 0.05–1 mg/mL. The highest peak (retention time 11.2 min) was used for the calibration curve. To perform GTN derivatization with fluorenylmethyloxycarbonyl (FMOC), 1 mL 30mM solution of FMOC chloride (dissolved in methanol) was added to 2 mL GTN solution prepared as described above, and the volume was brought to a total of 5 mL with HPLC mobile phase. The mixture allowed to react for 5 minutes at ambient temperature and 20 μL were injected into the chromatographic system.

2.5. PREPARATION OF TEST SAMPLES

Over 50 μL of the samples taken at different time intervals was added 1 mL 30 mM solution of FMOC chloride (dissolved in methanol), and the volume was brought to a total of 5 mL with the HPLC mobile phase. The mixture allowed to react for 5 minutes at ambient temperature and 20 μL were injected into the HPLC system.

2.6. TESTING THE ANTIBACTERIAL ACTIVITY OF THE IMPLANTS WITH GTN ADSORBED ON THE IMPLANT SURFACE

Nutrient agar (Mueller-Hinton) was poured into Petri dishes with a diameter of 100 mm, in a uniform layer of 4 mm. Inoculum preparation was performed by suspending 2-3 standard colonies in physiological serum. The culture medium must have a pH of 7.2-7.4 and a composition suitable for the proper development of the bacterial species to be tested. Deposition was achieved by flooding the nutrient medium with bacterial suspension, followed by removal of excess.

Drying of the inoculated plates was carried out by maintaining for 10 minutes at room temperature (22 °C ambient temperature) before the samples were deposited. The microorganisms to be tested came from standard reference strains, purchased from the Cantacuzzino Institute, being classified as sensitive to the action of the antibiotic of choice (GTN) [14].

To determine the MIC, was placed filter paper washers containing different amounts of GTN (2, 3, 4, 5, 6, 7, 8 μg) on culture media seeded with *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Petri dishes were incubated for 24 hours at 37 °C. MIC is the lowest concentration of antibiotics that caused inhibition of bacterial growth *in vitro* (Table 1).

Table 1. Classification of tested germs sensitivity to GTN

Microorganism test	**Antibiotic (control +)**	**R (mm)**	**IS (mm)**	**S (mm)**
Staphylococcus aureus	GTN	≤12	13-14	≥15
Escherichia coli		≤12	13-14	≥15
Pseudomonas aeruginosa		≤12	13-14	≥15

For testing the antibacterial effect, the diffusimetric method on nutrient agar (*Kirby-Bauer*), according to FR X, was used. The samples were deposited approximately 15 minutes after seeding, using an ophthalmological tweezers, applying each sample to be analyzed on the surface of the culture medium. The samples were deposited 1.5 cm away from the edge of the Petri box and 3 cm apart. Incubation was performed for 18 hours at 37 °C, in the inverted position of the Petri dish.

The reading of the results was performed using a graduated ruler, measuring the diameter of the inhibition zone (mm), induced by the test samples. The results were expressed as mean values obtained by performing the arithmetic mean of the corresponding diameters of three measurements. Very small colonies or subsequent invasion of the inhibition zone and discrete increases in the inhibition zone were not considered. The final results were expressed in sensitive, sensitive intermediate and resistant [15, 16].

3. RESULTS AND DISCUSSION

From Table 2 we can see that the highest amount of GTN was adsorbed by the HAp composite and the smallest amount was by HApTiCaFb. To quantify the amount of released GTN, a calibration curve was constructed in the concentration range of 0-1200 μg/mL, the equation of the straight line being: $y = 23947721\ x - 205991.9$ with a correlation coefficient $R^2 = 0.9962$ (Fig. 1).

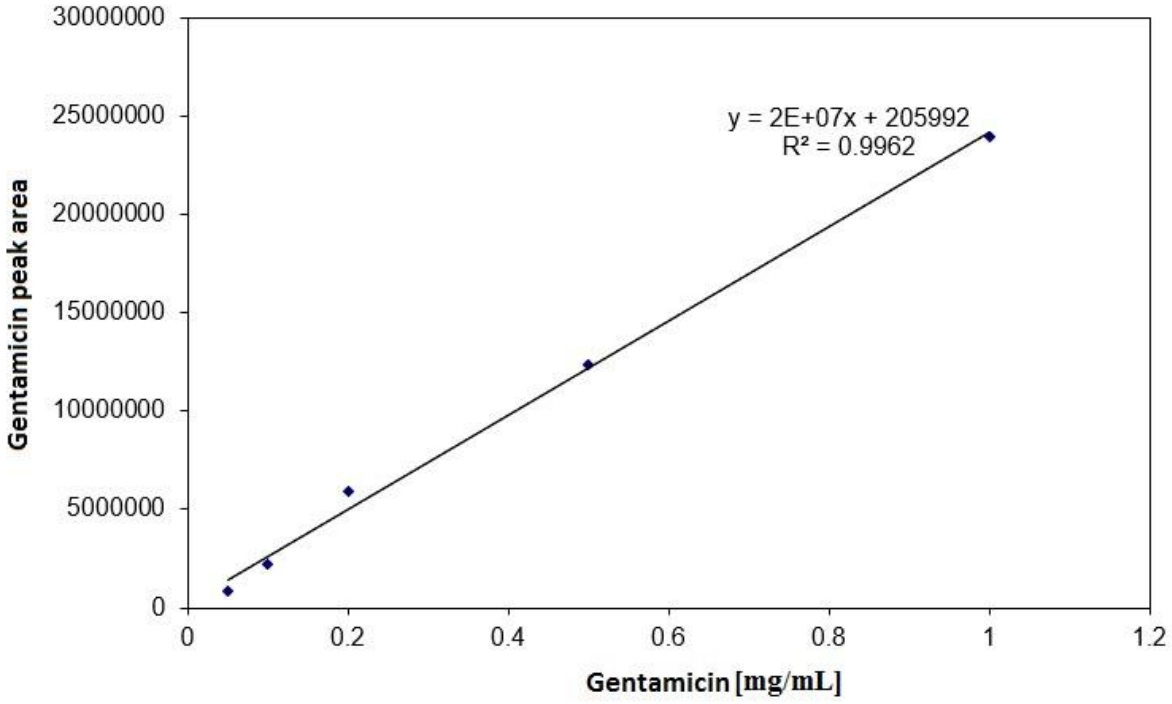

Figure 1. Calibration curve obtained for GTN [mg/mL]

Table 2. Weight of test implants before and after GTN chemosorption

Type of implant	Mass of implants before chemisorption [g]	Mass of implants after chemisorption [g]	The amount of GTN adsorbed [g]	Percentage of GTN adsorbed [%]
HAp	0.3912	0.4012	0.01	2.5562
HApTi	0.3955	0.4035	0.008	2.02271
HApTiFbCa	0.4221	0.4298	0.0077	1.8242

The retention time for GTN was about 11.2 minutes with a maximum absorption at 265 nm. From the graphical representation it can be observed that for HAp-GTN, the six-hour GTN release is close to 0, after six hours the release profile is exponential, with a spurt release between 144 h-216 h. In the case of HApTi-GTN, antibiotic release begins at 12 hours and occurs exponentially over a 14-day period. In the case of HApTiCaFb-GTN, GTN release is quantifiable 24 hours after immersion, the release profile being slow up to 48 hours. Between 48 hours and 72 hours there is a sudden release of GTN, a release stagnation occurs between 72 h-144 h and then a rapid release between 144 h-216 h (Fig. 2).

According to our experimental results, in the first 48 hours of the study, 45,226% of the GTN was released from the HAp-GTN compound, 33.919% from the HApTi-GTN and 2.583% from the HApTiCaFb-GTN. Then, the amount of antibiotic released increased to 70.854% for HAp-GTN, 80.402% for HApTi-GTN and 56.423% for HApTiCaFb-GTN up to 144 h (day 6). The final amount of GTN was released at a low speed between 288 h (12 days) and 336 h (14 days), being from about 97% to 100% (Fig. 3).

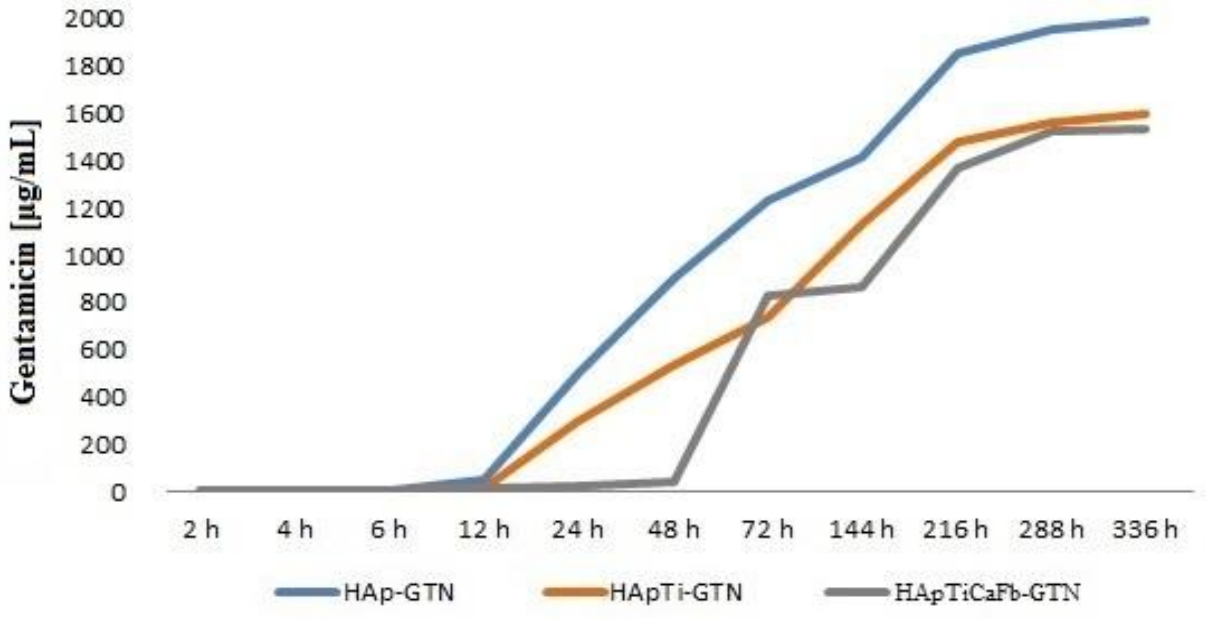

Figure 2. The amount of GTN released [µg / mL] from the implants tested in the time unit.

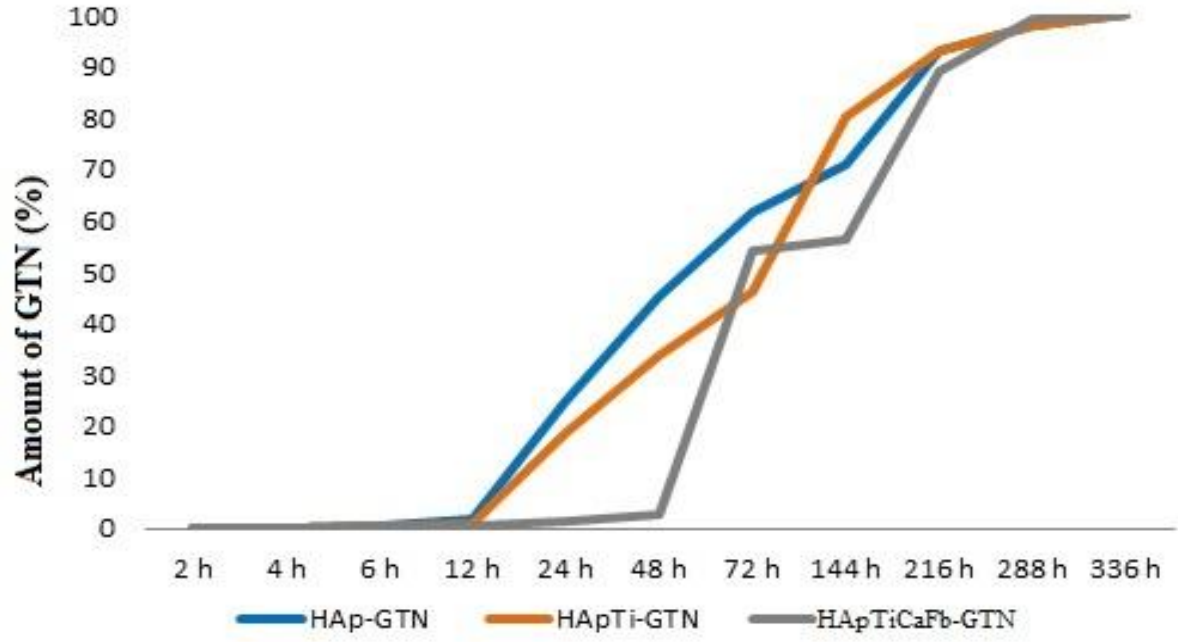

Figure 3. Percentage of GTN released in the time unit.

Following the development of antibiograms for the determination of MIC, we determined that the dose of 5 µg GTN is the smallest amount of antibiotic that causes the inhibition of bacterial growth on the tested germs. This concentration of antibiotic, established in the preliminary test, was used as a positive control in the subsequent antibiograms. Analysis of the antibacterial potential on tested bacterias showed that the efficacy of all implants is relatively equal to that of the pure antibiotic (Table 3). It can be seen that GTN binding to bioceramics does not influence the antibacterial activity, its release from becoming easily. HAp showed a weak antibacterial effect, the species *Staphylococcus aureus* being classified as resistant to it (14.5 cm) (Table 3). After performing the antibiogram, it was

observed that the diameters of the bacterial growth inhibition zones are approximately equal for the three tested implants (Table 3) [17, 18].

Table 3. Medium diameter area for bacterial growth inhibition

Sample		***S. aureus***	***E. coli***	***P. aeruginosa***
DZI (mm)	GTN (control +)	26.5	29.1	21
	HAp (control-)	14.5	16.5	8.5
	HAp-GTN	27.4	28.2	20.7
	HApTi-GTN	27.6	27.3	20.6
	HApTiCaFb-GTN	27.7	29.1	20.5

4. CONCLUSIONS

In the present study, the release of the chemisorbed antibiotic to the surface of the implants is made during 14 days, a sufficiently long period taking into account the chosen method of deposit. However, the simplicity of the chemisorption method in relation to the duration of the release constitutes favorable arguments for the use of antibiotic implants, made by the chemisorption method. The period of antibiotic release may be considered as sufficient to support osteointegration under antibacterial protection.

HAp-GTN, HApTi-GTN, HApTiCaFb-GTN implants showed antibacterial efficacy equal to that of pure GTN on the tested germs (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*). Following the development of antibiograms, we observed that HAp has medium antibacterial properties, *Escherichia coli* being classified as sensitive to its action, *Staphylococcus aureus* and *Pseudomonas aeruginosa* being resistant.

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