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BIOTEK REPORT NO: 2175-F

CONTRACT NO: DAMD17-92-C-2007

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CONTRACTING ORGANIZATION: Biotek, Inc.
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REPORT DATE: September 21, 1993

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13. ABSTRACT (Maximum 200 words)

This final report details the development, preparation, and characterization of biodegradable antibiotic beads for testing in animal models.

A process was developed for preparing 3-6 mm beads from antibiotics and biodegradable polymers. The process was used to prepare 200 3-6 mm beads from each of the following antibiotics: ceftriaxone, tobramycin, and placebo as well as a mixture of ceftriaxone and tobramycin for a total of 800 beads. The beads were delivered to the U.S. Army Medical Research and Development Command at Fort Detrick, Frederick, Maryland for evaluation in animal models. The beads were characterized by measurement of residual solvent levels, drug content, in vitro release, and in vitro bacterial inhibition against both gram positive and gram negative bacteria. The methods of development and results of in vitro studies are presented in the report.

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I. PURPOSE AND SCOPE OF THE RESEARCH EFFORT

A. Objectives

The objective of the work described in this report is to develop 3-6 mm biodegradable antibiotic beads for use in the prevention and treatment of osteomyelitis following bone surgery or open fractures. The beads are to be prepared from the antibiotics tobramycin and ceftriaxone in a biodegradable poly-lactide-co-glycolide polymer matrix. Antibiotic delivery in vitro is to be maintained at a level equal to the minimum inhibitory concentration for each drug for a period of 5-6 weeks. The polymer should degrade within a period of 6-8 weeks.

Past antibiotic beads and cements have suffered from a number of disadvantages, such as the use of non-degradable materials which must be removed in a second operation, the use of an antibiotic to which some bacteria are immune, and the use of designs which deliver too fast at early times and too slowly later on. All of these disadvantages can potentially be overcome by the approach presented here.

B. Military Significance

A major cause of morbidity and mortality in combat and battlefield conditions is infection of injuries, wounds, and burns. The need for protecting and treating troops against bacterial infection is recognized as important by the military and is a mission of the USAMRDC. Research in this area is needed to enhance self-preservation and the rapid return to duty of soldiers who have sustained non-life-threatening wounds and injuries. Of special interest to combat casualty care is the management and local treatment of osteomyelitis and contaminated open fractures using biodegradable sustained release antibiotic delivery systems **(see item B.2.e. of the Broad Agency Announcement - September 1989).**

In work complementary to that proposed here, U.S. Army Institute of Dental Research, USAIDR, has obtained promising in vitro and in vivo results with ampicillin-loaded biodegradable polylactide-glycolide microcapsules designed for topical treatment of wounds (**Setterstrom, 1984; Tice, 1986**), an application which relies on many of the same features as the use of larger antibiotic beads in bone cavities.

C. Background

The use of antibiotic-impregnated polymethyl methacrylate (PMMA) bone cement was first described by Buchholz in 1970 (see **Buchholz, et al., 1984** for review). Since then several investigators have confirmed the effectiveness of antibiotic bone cement mixtures for the treatment of open fractures, chronic osteomyelitis, total joint arthroplasty, and other deep wound infections (Wahlig, et al., 1984; Elson, et al., 1977; Buchholtz, et al., 1984; Calhoun and Mader, 1989). Antibiotic PMMA beads have been particularly useful for the treatment of chronic osteomyelitis Most clinical work started in Europe utilized gentamicin, because of the availability there of two gentamicin-PMMA commercial products: Septopal™ beads and Palacos™ bone cement (Martindale, 1989). In the United States beads containing tobramycin, vancomycin, and other antibiotics have been mixed in the operating room or pharmacy (Marks, et al., 1976; Fitzgerald, et al., 1983).

1. In Vitro Studies

Dissolution studies have shown antibiotic release from cement to depend on both the antibiotic and the type of cement. The rate and duration of release of gentamicin, oxacillin, and cefazolin from PMMA cement were found to be directly related to the amount of antibiotic added to the cement (**Marks, et al., 1976; Picknell, et al., 1977; Wahlig, et al., 1978**). All cases studied showed a first order decrease in release rate with time, an expected result when antibiotic is mixed with cement to form a monolithic structure whose delivery is controlled by a superficial depletion layer which thickens as drug is lost from the surface. For example, **Wahlig, et al. (1978)** found the initial release of gentamicin from one bead of PMMA to begin at 400-600 μg/day and fall to 10 μg/day by day 80. Similar results were obtained by **Von Frauhofer, et al. (1985)** with tobramycin-PMMA beads.

2. **Animal Studies**

In vivo studies have been conducted by several investigators with a variety of antibiotics and animals. After their in vitro release studies with oxacillin-impregnated bone cements, **Marks, et al. (1976)** followed the release in vivo after implantation of cement in the pelvis of mongrel dogs. Although blood levels peaked after 1 hour, they became non-measurable after 4 hours. Antibiotic levels in hematomas were bacteriostatic for 14 days, and the level in bone at 3 weeks was seven times the normal bone level after a parenteral dose. A similar study by **Wahlig and Dingeldein (1978, 1980)** of gentamicin release from PMMA in canine femurs showed bone levels of the antibiotic to be between 1.6 and 1.8 μg/g after 22 months.

Picknell, et al. (1977) implanted various antibiotic bone cements subcutaneously in mice and rats. In 24 hour in vitro tests from 2.5 to 10 percent of the antibiotic was released in active form, whereas cement plugs implanted in mice and rats produced only low blood concentrations. **Wahlig and Dingeldein (1980)** studied gentamicin bone cements in 14 beagle dogs after implantation in the medullary femur cavity. High concentrations of antibiotic were achieved in the hematoma, connective tissue and the cancellous bone close to the implant, particularly for the first 4-8 weeks after operation.

Fitzgerald (1983) developed an animal model to evaluate the treatment of infections by antibiotic PMMA. Osteomyelitis of the proximal portion of the tibia was consistently produced in the dog by instillation of a suspension of Staphylococcus aureus around an intramedullary foreign body made of acrylic bone cement. Gentamicin-impregnated cement prevented development of osteomyelitis in 9 of 10 dogs, whereas all control dogs developed osteomyelitis. Similar osteomyelitis model studies were conducted in rabbits by **Rodeheaver, et al. (1983)**.

3. **Clinical Studies**

Clinically, antibiotic impregnated PMMA bone cement and PMMA beads have been used for the prophylaxis of infection of total hip arthroplasty and for the treatment of infected arthroplasty sites (**Buchholz, et al., 1984; Wahlig, et al., 1984**). Several antibiotics were initially evaluated, and most investigators settled on gentamicin.

The use of two other antibiotics, tobramycin and vancomycin, has been explored recently Soto-Hall, et al. (1983) employed tobramycin-impregnated bone cement in the treatment of ten

patients undergoing revision of total hip arthroplasty. There were no immediate postoperative infections, and all patients were pain free and ambulatory without external support for two years. Eckman, et al. (1988) observed the level of antibiotics in 24 patients with compound fractures which were treated with tobramycin-impregnated PMMA beads. Drug levels in wound drainage and clots were significantly above the usual therapeutic range.

DeGroote, et al. (1979) treated 24 cases of osteomyelitis with 7 mm gentamicin PMMA beads implanted locally into curetted osteomyelitic cavities for 3 months. Nineteen of 25 cases responded well to the treatment. No controls were conducted for comparison. Calhoun and Mader (1989) reported unexpected results on treating 91 cases of adult osteomyelitis with tobramycin, vancomycin, and clindamycin beads. Antibiotic levels in seroma fluid remained above minimum inhibitory concentrations 1 month after administration.

Antibiotic beads have also been used for prophylaxis in abdominal and neck surgery, in elective colorectal surgery, and in the treatment of soft tissue infections in the perianal area and in the extremities.

4. Disadvantages of Polymethyl Methacrylate

There are a number of concerns regarding the use of antibiotic-loaded PMMA bone cement and beads. The most important is that they require a second operation for their removal, and they may reduce the mechanical strength of bone cement. They may release a large portion of antibiotic on the first day which may have harmful ototoxic and nephrotoxic effects. Enough antibiotic may remain trapped to encourage bacterial resistance or allergic reactions.

5. Biodegradable Antibiotic Bone Cement

Gerhart, et al. (1988) studied the release of gentamicin and vancomycin from cylinders of biodegradable bone cement of [poly(propylene fumarate) - methylmethacrylate] (PPF-MMA) implanted in rats for two weeks. The biodegradable PPF-MMA cement achieved and maintained a higher wound antibiotic level than did PMMA cement. However, only 15% of the cement resorbed after 3 months in rats, suggesting that complete resorption might take two years or longer. Moreover the polymer contained quantities of MMA monomer, benzoyl peroxide, and dimethyl-p-toluidine activator whose toxicity may adversely affect surrounding host tissues and impair host defenses.

Plaster of Paris has been used for filling bone defects because it is slowly resorbed (see Calhoun and Mader, 1989), and antibiotic-loaded plaster of Paris pellets have been studied in vitro (Mackey, 1982) for possible use against osteomyelitis.

6. Preliminary Studies at BIOTEK

a. Antibiotic Studies

Preliminary studies were conducted at BIOTEK before submission of the proposal with tetracycline PLA-PGA beads prepared by solvent evaporation. Tetracycline was selected for making the beads because of its availability and ease of assay. The beads were approximately 2 mm in diameter and contained 10.4 mg (25%) tetracycline per bead, with the balance being 75:25 polylactide-glycolide copolymer. The polymer was purchased from Birmingham Polymers, Inc., of

Birmingham, AL. In vitro release studies revealed that less than 10% of the drug was released from the beads during the first 50 days, a rate which was considered too slow. A second sample of beads released drug at a faster rate. The release profile showed a near zero-order release of tetracycline for 90 days.

The preliminary studies indicated that the solvent evaporation process is not useful for preparing particles larger than 2 mm in diameter, without significant reduction in the rate of release. At the 25% drug loading, the amount of polymer present forms a solid barrier which prevents rapid diffusion of tetracycline from the bead core.

b. Biodegradable Polymers

BIOTEK has been involved in the synthesis of biodegradable polylactide, poly-glycolide, polycaprolactone polymers and their copolymers for more than 15 years. Studies of the degradation of these polymers in vivo and in vitro have shown the rate of degradation to depend on polymer molecular weight and polymer composition. Recently, polymers of polylactide-co-glycolide (PLA-PGA) with a ratio of 50:50 of each polymer and also polymers of polylactide co-caprolactone (PLA-PCL) with a ratio of 90:10 were studied in vitro and also in vivo over a period of 15 weeks (105 days). The rate of polymer degradation was monitored by measurement of the change in polymer viscosity as a function of time. The results which are presented in Figure 1 show the rate of degradation both in vitro and in vivo to be very similar (compare the solid line with the dotted line in Figure 1). Also, there was a distinct difference in the rate of degradation between the two copolymers (compare the rate of degradation of PLA-PGA with PLA-PCL). On the basis of these results, we plan to determine the decrease in polymer viscosity in vitro for evaluating the rate of degradation of the copolymers used for preparing the antibiotic beads.

D. Selection of Antibiotics and Polymers

1. Antibiotics

A great deal of work has been published since 1970 by Buchholz and other European investigators on the use of PMMA bone cement and beads in combination with gentamicin antibiotic. Gentamicin was originally favored because of its potency against most bacteria. The subsequent emergence of resistant microorganisms such as Pseudomonas in some hospitals now limits the future value of gentamicin (**Goodell, et al., 1986**). BIOTEK will replace gentamicin with two antibiotics: tobramycin and ceftriaxone.

Tobramycin is one of seven antibiotics which have been incorporated into spherical anti-osteomyelitic implants; it is an aminoglycoside antibiotic with an antimicrobial spectrum similar to gentamicin, but it is two to four times more active against Pseudomonas aeruginosa. It has very low antigenicity and is well tolerated systemically in humans. Ceftriaxone is a third generation cephalosporin. It is effective against many infections and the drug of choice for bone and joint infections by many organisms. The drug is not orally effective and has to be administered intravenously (**Remington, 1990**). Table 1 shows the daily dose, therapeutic serum level, toxic serum level and minimum inhibitory concentration (MIC) for tobramycin and ceftriaxone and Table 2 illustrates the physical properties of the two antibiotics.

2. Biodegradable Polymers

The biodegradable polymer used in the beads must meet four requirements:

a. It must have FDA approval
b. It must fully degrade in the body soon after exhaustion of the drug
c. It must be absorbed in body tissue with little or no reaction
d. It must have little or no interaction with the antibiotic used

Among the potential candidates, polyesters prepared from lactic and glycolic monomers are prominent because they meet most of the requirements. These polymers have been shown to absorb harmlessly in body tissues (**Kulkarni, et al., 1966; Schindler, et al., 1977**). In vivo degradation of poly-D,L-lactide polymer in rabbits was shown to occur essentially by homogeneous erosion caused initially by random hydrolytic chain scission of ester groups, followed by loss of tensile properties as the molecular weight declines below 15,000. Hydrolysis is autocatalyzed by the liberated carboxyl groups. Enzymatic contributions to biodegradation were not found to be major

E. Antibiotic Dose and Duration of Release

1. Antibiotic Dose

There seems to be no general agreement about the dose of antibiotic to be mixed with beads. **Goodell, et al. (1986)** used 3 and 6 mm tobramycin beads weighing 12.8 mg and 160 mg, respectively. Each bead contained approximately 2% drug. Most PMMA studies have been conducted with beads prepared from a mixture of 2 g of antibiotic powder and 40-60 g of resin, the equivalent of 4% drug concentration (**Calhoun and Mader, 1989**). Optimum drug concentrations may be different for the proposed biodegradable beads.

For example **Sheftel and Mader (1986)** were able to arrest osteomyelitis in nine patients by administering a dose of 1.5 mg/kg of tobramycin every eight hours for 42 days, which is equivalent to the 3-5 mg/kg/day therapeutic dose of Table 1. This dose of tobramycin has been shown to produce therapeutic plasma levels of 4-10 μg/ml, and happens to be equivalent to the minimum inhibitory concentration (MIC) for the drug (0.06 - 8.0 μg/ml). In order to achieve 42 day release the rate of release should not exceed 1000/42 or 24 μg/mg/day, or 2.4% of the content of each bead per day if placed into an infinite sink. A 100 mg bead with 10% drug should release 240 μg of tobramycin per day. By varying the number and drug loading, the amount of drug released can be controlled

The variability in the volume, shape and vascularity of bone cavities makes selection of the total amount of drug and daily release rate for each bead somewhat difficult. In vitro studies will be used to assess the drug loading needed to achieve minimum inhibitory concentration of each antibiotic under sink conditions, even though little is known about the clearance rate from diseased bone cavities. A bone cavity is not an infinite sink, nor even a pharmacokinetically well defined environment with a specific rate of drug clearance; different bones, fractures and surgery will lead to different intracavity clearance rates. While the coating on BIOTEK's proposed beads will control the rate of drug delivery from each bead, the steady state concentration will also depend on the local clearance rate and on the number of beads used. In practice this will rely on the judgment of the surgeon in conjunction with published recommendations.

BIOTEK's purpose in Phase I is to develop beads which deliver a reproducible and therapeutically useful quantity of antibiotics for the specified period of time, as measured by an in vitro infinite-sink model similar to that employed by past workers in the field (e.g. Sastry, 1990).

2. **Duration of Antibiotic Delivery**

When **Sheftel and Mader (1986)** administered 1.5 mg/kg tobramycin to nine patients every 8 hours for an average period of 42 days, osteomyelitis was arrested in all nine patients after this period. **Tsukayama, et al. (1978)** implanted 60-90 six mm tobramycin-impregnated PMMA beads in 47 patients with infections. Bead therapy was 7-113 days with a mean of 37.7 days. In 80% of the cases the beads controlled the infection. **Wilson, et al. (1988)** studied the diffusion of tobramycin and cefotaxine from PMMA beads. Both antibiotics were released in quantities sufficient to inhibit the growth of bacteria in agar plates or broth culture over a 28 day period. These results suggest that release of a minimum inhibitory concentration of each antibiotic into an infinite sink for 42 days would be adequate to arrest osteomyelitis. This would be the initial target for duration of release. This target will be modified as new information becomes available from animal studies to be conducted by the US Army at the end of Phase I.

F. Methods

1. Procurement

a. Ceftriaxone and Tobramycin

The ceftriaxone and tobramycin were procured from Sigma Chemical Co. (St. Louis, MO). The respective lot numbers were 79F1018 and 60H04441.

b. Polymer

The polymers, Poly D,L-lactide-co-glycolide at ratios of 65:35 and 75:25, and a Poly L-lactide-co-glycolide at a ratio of 65:35 were procured from Birmingham Polymers, Inc. (Birmingham, AL). The lot numbers for the polymers were 104-45-1, 101-100-1, and 107-18-1, respectively. Table 3 is a list of the polymers evaluated.

2. Polymer Characterization

Two methods are used to characterize the molecular weight of the polymers. The reduced solution viscosity is a simple test, well suited for routine measurements. While viscosity and molecular weight are directly related, calculation of molecular weight from viscosity requires the knowledge of the Mark-Houwink coefficients for the type of polymer tested. Although such coefficients have been published for pure poly-L-lactide, the applicability to glycolide copolymers is not known. Gel permeation chromatography is a less ambiguous measure of the molecular weight and its distribution. It determines the size of solvated polymer molecules in comparison with polystyrene standards of known molecular weights. Assuming that the degree of solvation is the same for the test polymer and for polystyrene, molecular weights, as well as various molecular weight averages, can be obtained accurately.

a. **Reduced Solution Viscosity**

Reduced solution viscosity is determined as a measure of the molecular weight of the polymer. The polymer is dissolved in hexafluoroisopropanol at a concentration of 1.0 mg/ml. This is filtered into a size 50 Cannon-Fenske viscometer maintained at 30°C by a water bath. The flow time of the solution (t_s) is determined in quintuplicate and related to the flow time obtained with pure solvent $t(t_o)$. Standard deviations of the replicate flow times are typically below 0.2 seconds. The reduced solution viscosity (dl/g) is calculated from the formula:

$$\frac{t_s - t_o}{0.1 \times t_o}$$

b. **Gel Permeation Chromatography**

Gel permeation chromatography (GPC) was used also to test for the molecular weight of the polymer. It was performed at Research Triangle Institute (RTI), Research Triangle Park, North Carolina. The polymer was dissolved in chloroform and the solvent passed through a mixed bed column. After injection of the polymer solution, the eluted fractions were monitored on a refractive index detector. By means of a calibration run with accurately identified polystyrene standards, a molecular weight value assigned to each fraction.

c. **Polymer Composition**

The monomer ratios and presence of impurities was determined by NMR spectroscopy and were performed at Research Triangle Institute, Research Triangle Park, North Carolina, using a Brucker Model Wm-250 Supercon spectrometer. The polymer samples were dissolved in $CDCl_3$ and the resonances corresponding to the various H atoms in the polymer were recorded.

d. **Polymer Crystallinity**

Differential scanning calorimetry (DSC) measurements are used to estimate the degree of crystallinity of polymers. The tests were performed at Research Triangle Institute, Research Triangle Park, North Carolina. The polymer samples were heated at a constant rate and the absorption of heat (endotherm) or liberation of heat (exotherm) by the sample in comparison to an inert reference indium standard was recorded.

G. Microsphere Preparation

1. **Solvent Evaporation (Oil in Water)**

In the solvent evaporation process a solution of the drug and polymer in methylene chloride is emulsified in water in the presence of polyvinyl alcohol. The oil in water emulsion is stirred until most of the solvent has evaporated and the microspheres harden. Preparing microspheres by this method requires the drug to be soluble in methylene chloride and insoluble in water; otherwise most of the drug will be lost to the aqueous phase leaving only a small suspension of drug particles in the microspheres. We previously utilized the solvent evaporation process for preparing micro-

spheres from water soluble drugs such as Vitamin B12. Despite the high aqueous solubility of the vitamin the process proved to be very useful because of the low cost of the drug and its very low 10 μg/day dose. The high cost of ceftriaxone and tobramycin and its relatively high daily dose precluded the use of solvent evaporation for preparing antibiotic beads.

2. Solvent Evaporation (Water in Oil)

A 400 ml solution of 1.23% Span 85 was prepared by adding 5 ml of Span 85 in 400 ml of corn oil. The solution was stirred with an impeller mixer (Lightnin Mixing Equipment Co., USA) set at 1050 rpm.

1.0 gram of ceftriaxone (Sigma, St. Louis, MO) was dissolved in 7 ml of distilled water (Poland Spring, ME). Nine grams of calcium sulfate (Fisher Scientific, Medford, MA) were carefully weighed out and added to the aqueous drug solution to form a "slurry".

The aqueous drug/$CaSO_4$ mixture was poured into the oil phase solution and stirred at 1050 rpm. After two minutes, the speed of the mixer was reduced to 850 rpm and after four hours of mixing at 850 rpm, the solution was poured through 300 μm and 25 μm sieves to collect all microspheres in the 25-300 μm range. The microspheres were collected on a Buchner Funnel using Whatman No. 4 filter paper, washed with 100 ml of ethyl acetate to facilitate the removal of processing oil and dried overnight. A list of the microsphere batches prepared is given in Table 4 as MS1 to MS3.

3. Spray Coating

We have evaluated spray coating of ceftriaxone onto pure polymer beads. The first step in the process is to prepare 1-2 mm **pure polymer beads** to serve as core. The pure polymer beads were prepared by solvent evaporation of an emulsion of a polymer solution in distilled water in the presence of polyvinyl alcohol. Three batches of beads were prepared from the three copolymers listed in Table 3.

The pure polymer core prepared from both 65:35 PLA-PGA copolymers was rejected because the core particles were too soft and could not hold their shape. The pure polymer core prepared from 75:25 PDLLA-PGA was selected for further study because of their strength and ability to maintain their shape.

The pure polymer beads were prepared as follows:

In a typical procedure, 15 grams of polymer were dissolved in 50 ml methylene chloride. The solution was slowly poured into 2000 ml of a 2% solution of polyvinyl alcohol in distilled water and the mixture was stirred at a rate of 250 rpm. After 18 hours the temperature of the emulsion was slowly raised at a rate of 0.5 degrees per minute for a period of approximately 1 1/2 hours and until the temperature reached 65°C. The emulsion was left at that temperature for a period of 0.5 hours and then allowed to cool to room temperature. The hardened beads were then filtered, and washed 5 times with distilled water.

The second step in preparing antibiotic beads is to spray an aqueous solution of ceftriaxone onto the pure polymer beads to form **spray core microsphere beads** (Figure 2A). Before spraying, several binders were considered as additives to the spray solution in order to improve the adhesion and film properties of the antibiotic coating. The binder must be carefully selected in order to meet the following requirements:

a. inert
b. Nontoxic
c. Does not interact with the drug or polymer
d. Does not affect the integrity of the microcapsule
e. Does not affect the release of the drug from the microcapsule
f. Approved by the FDA for parenteral use
g. Water soluble
h. Film forming

Work was conducted with dextrose which is one of few candidates meeting nearly all the above criteria. The spray solution was prepared from ceftriaxone and dextrose at a ratio of 80% drug to 20% dextrose. In a typical procedure ceftriaxone and dextrose USP were dissolved in distilled water. 75:25 PDLLA-PGA microspheres were then charged into the BIOTEK microfluidized bed unit and coated with this solution at a rate of 0.25 ml per minute until the target coating level was applied.

The third step in the process is to apply a final coating of pure polymer to form **spray core microcapsule beads.** The purpose of the coating is to control the rate of drug release from the beads (Figure 2B). After removing a representative sample of the spray core for assay and release study, the remaining core was returned to the microfluidized bed unit for overcoating with polymer. A 1% polymer solution was prepared by dissolving 1 g of 75:25 PDLLA-PGA in 100 ml acetone. The solution was then sprayed over the ceftriaxone/dextrose coated beads (Batch SC2) at a rate of 0.6 ml per minute. Approximately 42 ml of the polymer solution was applied for a theoretical coating of 2.1 %. The beads were then removed from the coating chamber and dried at room temperature before characterization.

4. Preparation of Porous Microspheres

Because of the weight of the beads, spray coating was not a practical method of loading ceftriaxone onto the beads. A possible remedy to this situation was the preparation of porous microspheres. The average weight of a 75:25 PDLLA-PGA microsphere (approximately 2 mm diameter) is 2.9 mg. The average weight of a porous microsphere of the same polymer type and size is 1.35 mg, a weight difference of 53.4%. Figure 3 is a cross sectional view of a porous microsphere.

H. Bead Preparation by Marumerization

Marumerization is a process which is used to prepare beads from a dough of polymer and antibiotic. The dough is first extruded into small cylindered pellets which are then shaped into spheres using a marumerizer. For these studies we assembled a small bench scale marumerizer (Figure 4).

The first stage in preparing marumerized pellets is to develop a solvent system for the polymer which allows the formation of a dough-like mass which can be kneaded and shaped as desired. BIOTEK had previously developed a solvent system which allows the formation of a moldable dough from PLA-PGA biodegradable polymers. The solvent system is proprietary, but is based on totally innocuous and FDA acceptable solvents for parenteral use.

In previous studies we used the solvent system to prepare "dough" from PLA-PGA polymer with ratios of 75:25 and 85:15. In this project, we repeated the tests with the 75:25 polymer and the two 65:35 polymers listed in Table 3. The tests with both 65:35 polymers were not successful. The dough from these polymers was rolled into spheres, but the spheres remained soft and never hardened, most likely due to retention of the solvent by the polymer.

The dough prepared from 75:25 PDLLA-PGA was better than the dough from the other two polymers. Approximately 1 ml of solvent was required for 0.25 of polymer. To this dough 0.25 g of ceftriaxone was added and the mixture kneaded until uniform. The mixture was rolled into a cylinder and small 5 mm pellets were cut. After preliminary hand rolling (with gloves), the beads were placed in the lab scale marumizer and rotated until the edges were smooth and the beads hardened.

1. Marumerization of Beads with Drug Powder

A dough was prepared from 75:25 PDLLA-PGA. To this dough 0.25 g of micronized ceftriaxone powder was added and the mixture kneaded until uniform. The mixture was rolled into a cylinder and small 5 mm pellets were cut. After preliminary hand rolling (with gloves), the beads were placed in the lab scale marumerizer and rotated until the edges were smooth and the beads hardened. The beads were then removed and coated with polymer by dipping three times in a 10% solution of 65:35 PDLLA-PGA in acetone or by spindle spraying with a 10% polymer solution to coat the beads.

2. Marumerization of Beads with Drug Microspheres

A dough of 65:35 PDLLA-PGA polymer was prepared. The dough was rolled on a clean glass plate and 3.2 grams of antibiotic/$CaSO_4$ microspheres was evenly distributed in the dough. The dough was then kneaded manually to insure even drug distribution then rolled into a cylinder, and 4-6 mm pellets were cut from the cylinder. The pellets were then rolled and placed in a lab scale marumerizer to prepare the spheres. After a spherical shape was obtained, the beads were hardened in a low-vacuum freezer for 72 hours.

3. Marumerization of Beads with Drug Microspheres and Coated with Drug Powder

The pellets were prepared as described above by mixing the microspheres with the polymer dough and adding micronized ceftriaxone powder to coat the beads. This reduces their tackiness and increases the overall antibiotic level.

I. Characterization of the Antibiotic Beads and Microspheres

The characterization of the antibiotic beads includes antibiotic content, residual solvent, size distribution, surface morphology, in vitro release antibiotic profile, and in vitro bacterial efficacy.

1. Antibiotic Content of Microspheres

Ceftriaxone - Approximately 10 mg of ceftriaxone microspheres were weighed into each of three clean 50 ml volumetric flasks and filled to the mark with distilled water. The flasks were then placed in a sonicator for 15 minutes to insure total dissolution of the microspheres. The solution was then read in a 1 cm^3 quartz curvette at 242 nm. The concentration of the drug was calculated from a calibration curve (Figure 4).

Tobramycin - A homogeneous enzyme immunoassay kit for tobramycin detection was purchased from Syva (San Jose, CA). Approximately 10 mg of tobramycin microspheres were weighed into each of three clean 100 ml volumetric flasks and filled to the mark with distilled water. The flasks were then sonicated for 15 minutes to facilitate dissolution of the microspheres. A 1.0 ml aliquot was taken from each sample and the reagents were added. The solutions were read at 340 nm on a Milton Roy Spec 21 spectrophotometer and the concentrations were calculated from a calibration curve (Figure 5).

2. Antibiotic Content of Beads

The ceftriaxone beads were assayed for drug content as follows. Each bead was weighed and placed in a 50 ml volumetric flask, together with 10 ml of methylene chloride to dissolve the polymer, and 25 ml of distilled water to dissolve the ceftriaxone. The flask was then shaken until the aqueous and methylene chloride phase was clear. After separation of the two phases, the aqueous phase was removed and read in a 1 cm^3 quartz cuvette at 242 nm. The concentration of the drug was calculated from a calibration curve prepared in distilled water.

3. Residual Solvent Levels of Beads

Although the preparation of antibiotic beads requires a solvent system which is 75% aqueous, a method of residual solvent detection was developed to determine the level of residual acetone.

Total acetone was quantitated on a solution of antibiotic beads in optima grade chloroform (Fisher Scientific, Medford, MA) using a Perkin Elmer Sigma 2B gas chromatograph with a Hewlett-Packard integrator. The column is a 10% AT-1000 with Chromasorb W/AW (Alltech Associates, Deerfield, IL) on 80/100 support 6' x 1/8" stainless steel. The initial temperature is held at 60°C for one minute and is increased to 110°C at a rate of 5°C/minute in order to optimize separation. A flow injection detector is used with helium as a carrier gas at 40 psig. The injection volume is 2 μl of the antibiotic bead solution. The concentrations are calculated from a calibration curve prepared with G.C. grade acetone prepared in chloroform (Figure 6).

4. Size Distribution

a. Antibiotic Microspheres

Microsphere size distribution for each run was determined by sieve analysis through a 75 μm and a 300 μm sieve using an Allen-Bradley sonic sifter to insure complete separation of the microspheres.

b. **Antibiotic Beads**

The marumerized beads were prepared by hand and their diameter was measured directly using a metric ruler. After 72 hours of vacuum freezing they were sieved through Gilson sieves of the following size fractions: 6.3 mm and 2.8 mm.

c. **Scanning Electron Micrographs (SEM's)**

Scanning electron micrographs of select runs were taken at Analytical Answers, Inc., Woburn, MA using an ISI "Topcon" Model DS-130.

5. **In Vitro Antibiotic Release**

The following general procedure was employed to determine the release of ceftriaxone from the beads. An accurately weighed amount of beads was placed in an L-shaped tube with 40 ml of pH 7.4 phosphate buffered saline (PBS) elution medium. The tube was then placed in a 37°C shaker bath and shaken laterally at 100 rpm. The elution medium was removed at periodic intervals and replaced with fresh buffer solution. The concentration of ceftriaxone was determined by measurement of UV absorption of the elution medium at a wavelength of 242 nm in a 1 cm^3 quartz cell using PBS as a blank.

Tobramycin release was determined as described above, with the use of o-pthaldialdehyde, and spectrophotometrically read at 340 nm.

6. **In Vitro Bacteriological Evaluation**

Staphylococcus aureus, ATCC No. 29996 (American Type Culture Collection, Rockville, MD) culture was evenly streaked on Mueller-Hinton II with 5% sheep blood Agar plates (BBL/CMS, Wilmington, MA). Two antibiotic cylinders were placed on each blood plate and incubated at 37°C for 18 hours. The circular zone of inhibition was measured along three different diameters and the diameter of the cylinder subtracted from each measured zone. The mean was calculated and divided by two to give the width of the inhibition annulus. The cylinder was then rotated 180°C and transferred to a freshly streaked plate, and the process was repeated until no inhibition was observed (Figure 7).

Since ceftriaxone is not effective in the inhibition of certain gram negative bacteria, blood agar plates were streaked with P. aeruginosa ATCC #27853 in order to determine the efficacy of tobramycin sulfate beads.

II. RESULTS

A. Polymer Characterization

The properties of the polymers are presented in Table 3. The following is a discussion of the properties.

1. Viscosity

The inherent viscosity of the 65:35 PDLLA-PGA was determined to be 0.61 dl/g, the inherent viscosity of the 75.25 PDLLA-PGA was determined to be 0.80 dl/g, and the inherent viscosity of the 65:35 PLLA-PGA was 1.09 dl/g.

2. **Gel Permeation Chromatography (GPC)**

The molecular weight of the 65:35 PLLA-PGA was determined to be 99,600 daltons, the molecular weight of the 65:35 PDLLA-PGA was determined to be 50,000 daltons, and the molecular weight of the 75:25 PDLLA-PGA was determined to be 129,000 daltons.

3. **Nuclear Magnetic Resource (NMR)**

The ratio of the copolymers was determined to be 65:35 PDLLA-PGA, respectively. For the 65:35 DL PLA-PGA, the ratio of the copolymers was determined to be 78:22 PDLLA-PGA, respectively, and for the 75:25 PDLLA-PGA the ratio of the copolymers was determined to be 64.36 PLLA-PGA, respectively for the (L-) PLA-PGA.

4. **Differential Scanning Calorimetry (DSC)**

The DL polymers were amorphous and the L-polymer was crystalline.

B. Preparation of Microspheres

Microsphere batches were prepared from ceftriaxone and tobramycin. The batches and their composition are listed in Table 4. The average content for each microsphere batch is presented in Table 4.

Three batches of antibiotic microspheres were prepared using the water in oil evaporation process. They were numbered MS1, MS2, and MS3, where MS1 is a ceftriaxone microsphere, and MS2 is a tobramycin microsphere batch. The loading ranged from 8.2% to 9.2%. Assays of the batches are given in Table 4. MS3 was a placebo batch with no drug.

1. **Size Distribution of Microspheres**

Figure 8 illustrates the size distribution of both batches of microspheres, MS1 and MS2. Size distribution is controlled by varying different parameters of microsphere preparation, i.e stirring speed, concentration, volume, number of baffles, etc. Size distribution analysis of batches MS1 and MS2 showed more than 90.6% and 93.2% of the microspheres to be in the desired size range 75-300 μm, respectively (Figure 8).

C. Preparation of Antibiotic Beads

Eleven batches of beads were prepared (MB1 through MB7) for preliminary testing. Batches MB8 through MB11 represent the batches that were submitted to USAIDR for testing in animal models. These batches were fully characterized as described below.

1. Size Distribution of Beads

The marumerized ceftriaxone beads were prepared by hand and their diameter was measured directly using a metric ruler. After 72 hours of vacuum freezing they were sieved through Gilson sieves of the following size fractions: 6.3 mm and 2.8 mm.

The tobramycin, dual antibiotic and placebo beads were also sieved through 6.3 mm and 2.8 mm Gilson sieves. All three, 90.2%, 93.0%, and 91.1% were within specifications, respectively.

D. Residual Solvent

The ceftriaxone beads were assayed in triplicate and the total parts per million of acetone were 427±8 ppm.

The tobramycin beads were assayed in triplicate and the residual acetone levels were 520 ppm ± 7. The placebo beads were assayed in triplicate and their acetone content was determined to be 610 ppm ± 8. The dual antibiotic beads were assayed in triplicate and their acetone level was determined to be 425 ppm ± 5.

E. Preparation of Antibiotic Beads for Animal Testing by USAMRDC

1. Introduction

Several batches of beads were prepared in fulfillment of the contract requirement. On August 16, 1993, the following four batches of 3-6 mm antibiotic beads were shipped to Dr. Elliot Jacob at USAIDR for evaluation in animal models.

Batch No.	Antibiotic	Size	Number of Beads
MB9	Ceftriaxone	3-6 mm	200
MB10	Tobramycin	3-6 mm	200
MB11	Placebo	3-6 mm	200
MS8*	Ceftriaxone plus Tobramycin	3-6 mm	200

The beads were prepared by the processing techniques described in the methods section. The properties of the beads are described in the following sections.

2. Assay of Antibiotic Microspheres

The assay of the antibiotic microspheres and beads was performed as described in the Methods section. The ceftriaxone microspheres were assayed and found to contain 9.2% drug, while the tobramycin microspheres were determined to contain 8.2% drug (Table 4).

3. Assay of Antibiotic Beads

The ceftriaxone beads were assayed and found to contain 4.3% antibiotic, while the tobramycin beads were found to contain 8.50% antibiotic. The high level of antibiotics in the beads in comparison to the microspheres is caused by rolling the beads in antibiotic powder during the preparation process as described under Methods. The dual antibiotic beads were assayed and found to contain 2.86% ceftriaxone and 2.50% tobramycin.

Figure 9 shows the release of ceftriaxone from beads (MB9) prepared from ceftriaxone/$CaSO_4$ microspheres coated with micronized ceftriaxone powder. Figure 9 illustrates a high dose burst of 44% within one day with a relatively linear release of 85% over 38 days. The duration of release is estimated to be 60 days.

Figure 10 shows the release of tobramycin from MB10 beads. A burst of 53% was observed initially followed by sustained release for 35 days. The duration of release is estimated to be 40 days.

4. In Vitro Bacteriological Evaluation of Deliverables

Figure 11 is a photograph of the zone of inhibition after 12 days provided by ceftriaxone beads from Batch MB9 against S. aureus.

Figure 12 shows the zone of inhibition as a function of time of MB9 antibiotic beads against S. aureus ATCC #29996. The duration of inhibition was 45 days.

Since ceftriaxone is not effective in the inhibition of certain gram negative bacteria, blood agar plates were streaked with P. aeruginosa ATCC #27853 in order to determine the efficacy of tobramycin sulfate beads. P. aeruginosa was used as a test gram negative bacteria due to the inability of the B. subtilis bacteria cultures to remain viable and uncontaminated. Figure 13 illustrates the efficacy of antibiotic bead batches MB9 against Pseudomonas aeruginosa. Pseudomonas aeruginosa was effectively inhibited for 21 days.

Figures 14 and 15 show the zones of inhibition of both S. aureus and P. aeruginosa by the dual antibiotic beads (MB8). S. aureus was inhibited for 28 days and P. aeruginosa was inhibited for 23 days, respectively.

III. CONCLUSIONS

BIOTEK, Inc. has developed ceftriaxone beads, tobramycin beads, and a combined ceftriaxone and tobramycin bead that release therapeutic levels of the antibiotics and inhibit in vitro bacterial growth for 21 to 45 days. The beads are approximately 3-6 mm in diameter and consist of the antibiotic encapsulated in microspheres which are dispersed throughout a biodegradable lactide-glycolide polymer.

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TABLE 1

ANTIBIOTIC PROPERTIES

	PROPERTY		SERUM CONCENTRATION		
ANTIBIOTIC	MIC * (µg/ml)	DAILY THERAPEUTIC DOSE	THERAPEUTIC LEVEL (µg/ml)	ACUTE TOXIC LEVEL (µg/ml)	CHRONIC TOXIC LEVEL (µg/ml)
Tobramycin	0.06–8.0	3–5 mg/kg	4–10	>12	2
Ceftriaxone	E. Coli .016 – .5 S. aureus 1–2 P. aeruginosa 8–64	1–2 g/day	10–250	–	–

* MIC = Minimum Inhibitory Concentration

130,75–1tbl

TABLE 2

PHYSICAL PROPERTIES

PROPERTY	TOBRAMYCIN	CEFTRIAXONE
Molecular Weight	467.54	554.58
M.P. (°C)	217	155*
Solubility in water	20%	40%
Solubility in ethanol	0.05%	Very slightly soluble
pH of 1% Solution	--	6.7
UV Absorption (nm)	None	242 and 272

* Decomposes

130,75-1tb2

TABLE 3

PLA–PGA POLYMERS

Type	Poly(DL–lactide–co–glycolide)		Poly(L–lactide–co–glycolide)
BPI Lot No.	104–45–1	101–100–1	107–18–1
Monomer Ratio	65/35	75/25	65/35
Inherent Viscosity (dl/g)	0.61	0.80	1.09
Copolymer Composition – (^{1}H–NMR)	65/35	78/22	64/36
Molecular Weight	50,000	129,500	175,300

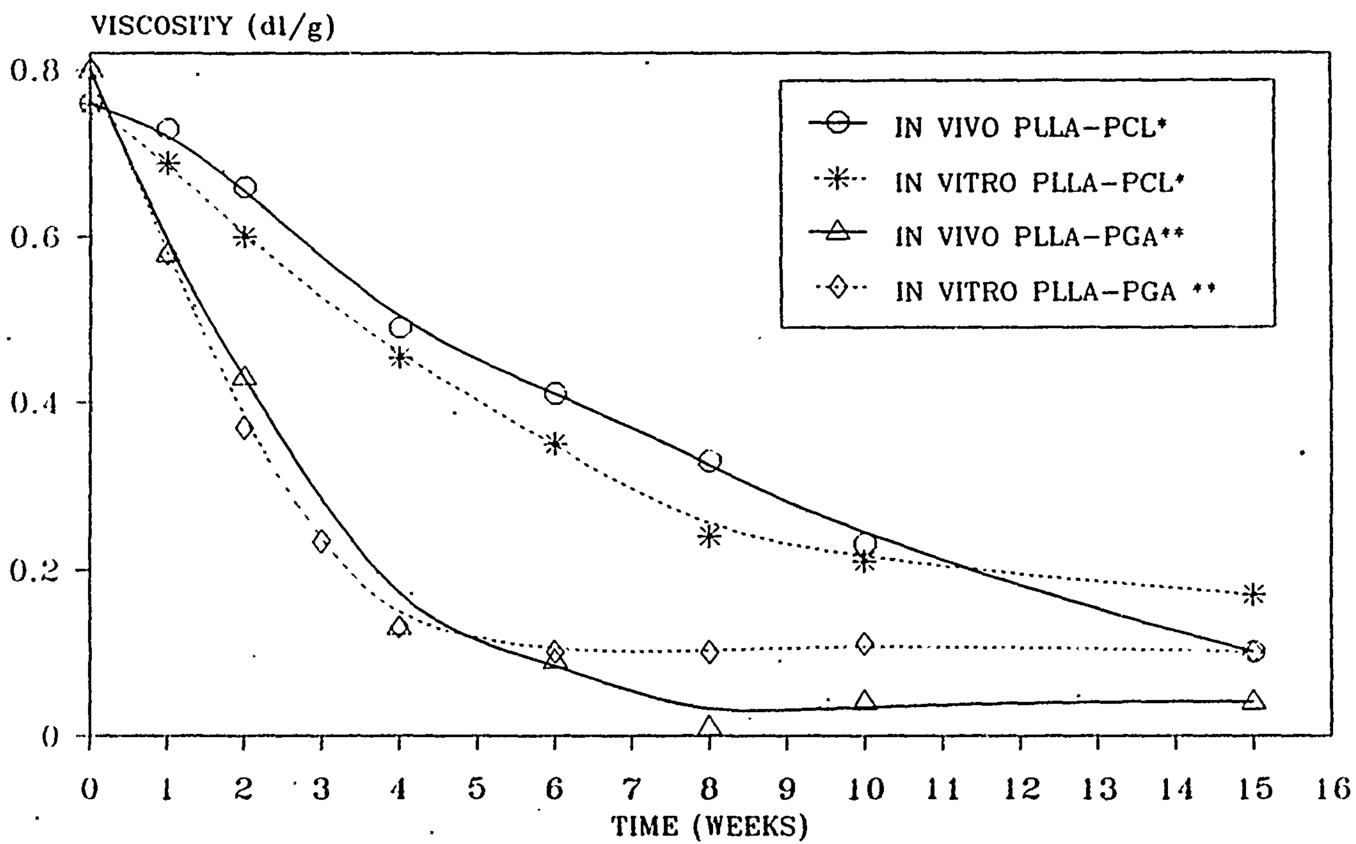
TABLE 4

ANTIBIOTIC BEADS

Batch No	Particle Type			Polymer/Additive		Overcoat Dipping Conc.	Core		Ceftriaxone Content	Tobramycin Content	Inhibition of S. aureus (Days)	Inhibition of P. aeruginosa (Days)
	Antibiotic	Type	Size	Type	Composition		Material	Size				
MS1	Ceftriaxone	Microsphere	25–300 μm	$CaSO_4$	$CaSO_4.2H_2O$				9 2%			
MS2	Tobramycin	Microsphere	25–300 μm	$CaSO_4$	$CaSO_4.2H_2O$					8.2%		
MS3	Placebo	Microsphere	25–300 μm	$CaSO_4$	$CaSO_4.2H_2O$							
MB1	Ceftriaxone	Marumerized Bead	3–5 mm	PDLLA–PGA	75:25		Pure Drug	Powder	4.4%		4	
MB2	Ceftriaxone	Marumerized Bead	3–5 mm 5–6 mm	PDLLA–PGA PDLLA–PGA	65:35 65:35	10%	Pure Drug	Powder	1.0%		10	
MB3	Ceftriaxone	Marumerized Bead	3–5 mm 5–6 mm	PDLLA–PGA PDLLA–PGA	75:25 65:35	10%	Pure Drug	Powder	1.18%			
MB4	Placebo	Marumerized Bead	3–5 mm 5–6 mm 5–6 mm	PDLLA–PGA PDLLA–PGA PDLLA–PGA	75.25 65:35 65:35	10% Sprayed 20%	Pure Drug	Powder	1.18%		10	
MB5	Ceftriaxone	Marumerized Bead	3–6 mm	$CaSO_4$	$CaSO_4.2H_2O$		Pure Drug	Powder	9.2%		30	
MB6	Ceftriaxone	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS1	75–300 μm	2.16%		30	
MB7	Ceftriaxone/ Tobramycin	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS1 MS2	75–300 μm 75–300 μm	2.16%	2.14%	27	24
MB8	Ceftriaxone/ Tobramycin	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS1 MS2	75–300 μm 75–300 μm	2.86%	2.5%	28	23
MB9	Ceftriaxone	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS1	75–300 μm	8.5%		45	
MB10	Tobramycin	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS2	75–300 μm		4.3%		21
MB11	Placebo	Marumerized Bead	3–6 mm	PDLLA–PGA	65:35		MS3	75–300 μm	0%	0%	0	0

FIGURE 1

POLYMER VISCOSITY AFTER AQUEOUS EXPOSURE OF MICROSPHERES AND IMPLANTATION OF COATED BEADS IN RABBITS



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* 90/10 ** 65/35

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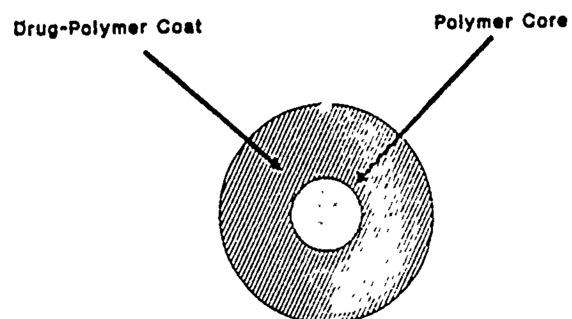
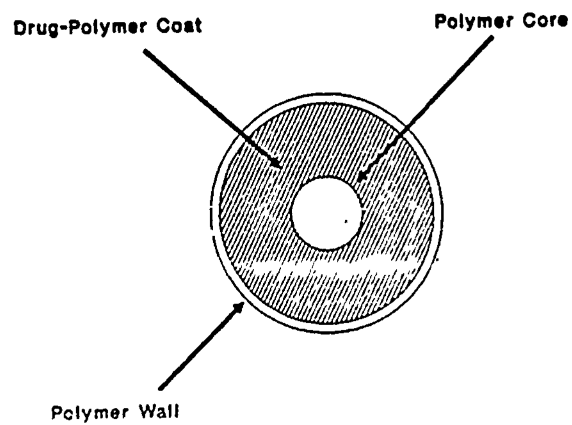
FIGURE 2

A
SPRAY CORE MICROSPHERE



3#SPRACOR1

B
SPRAY CORE MICROCAPSULE



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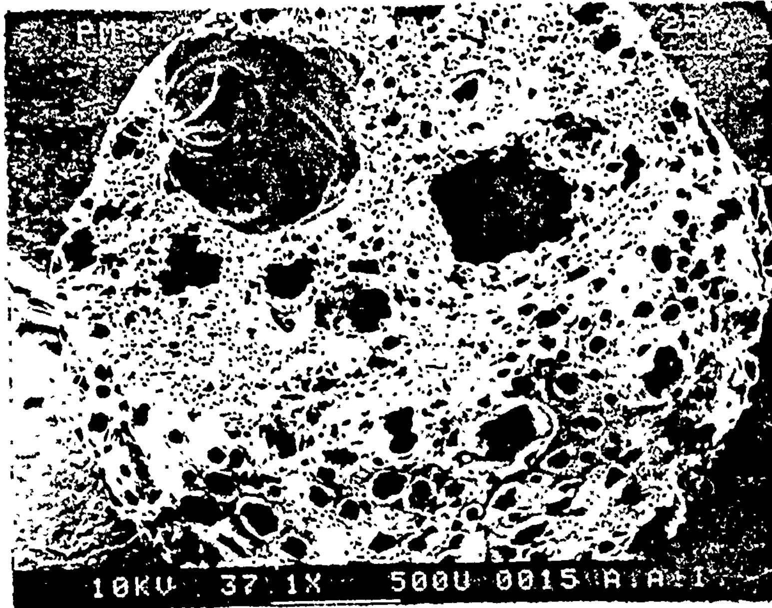


FIGURE 3 - Scanning Electron Micrograph X-Section of Porous Bead. Magnification 37.1X.

FIGURE 4

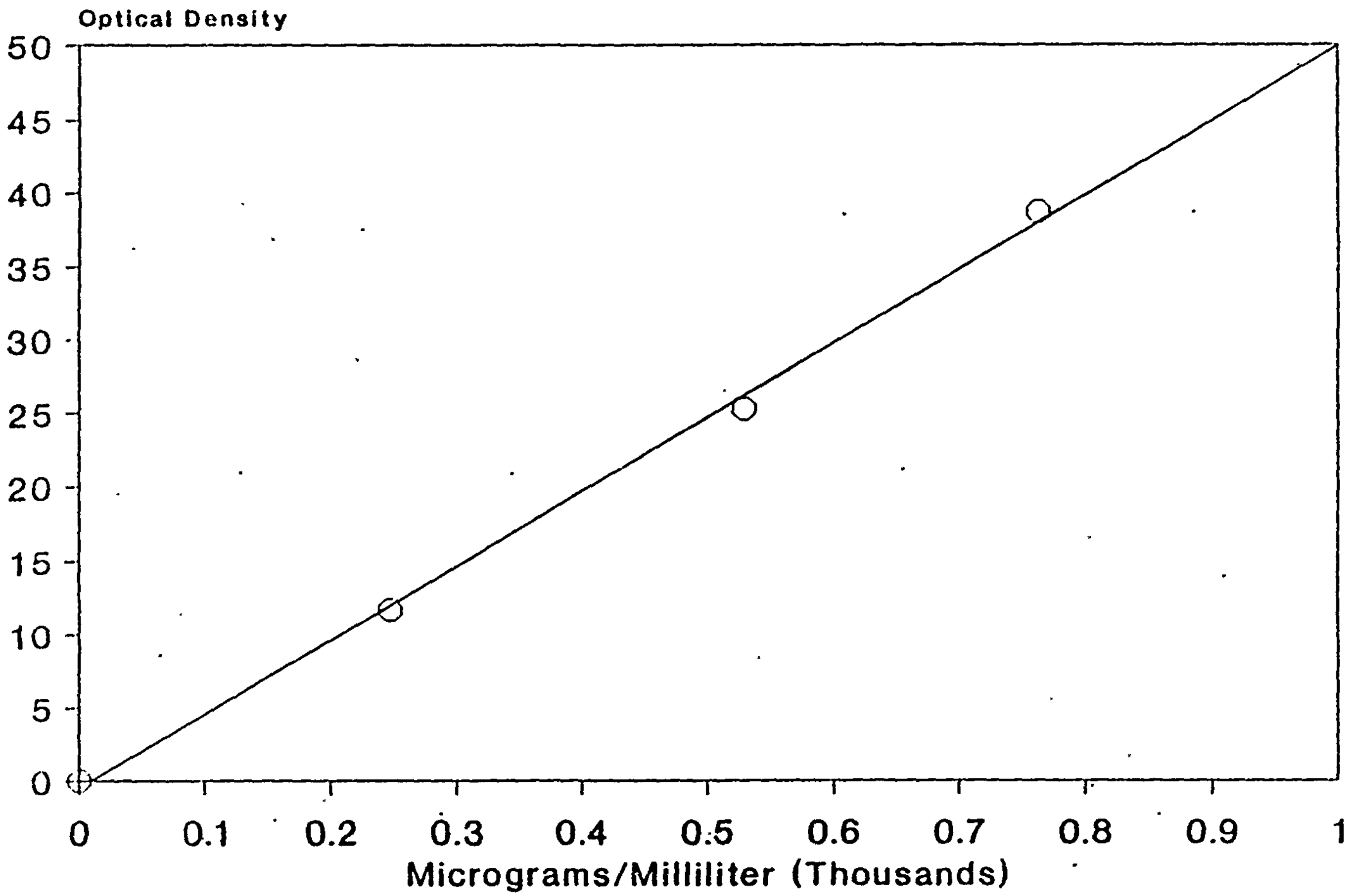


FIGURE 5

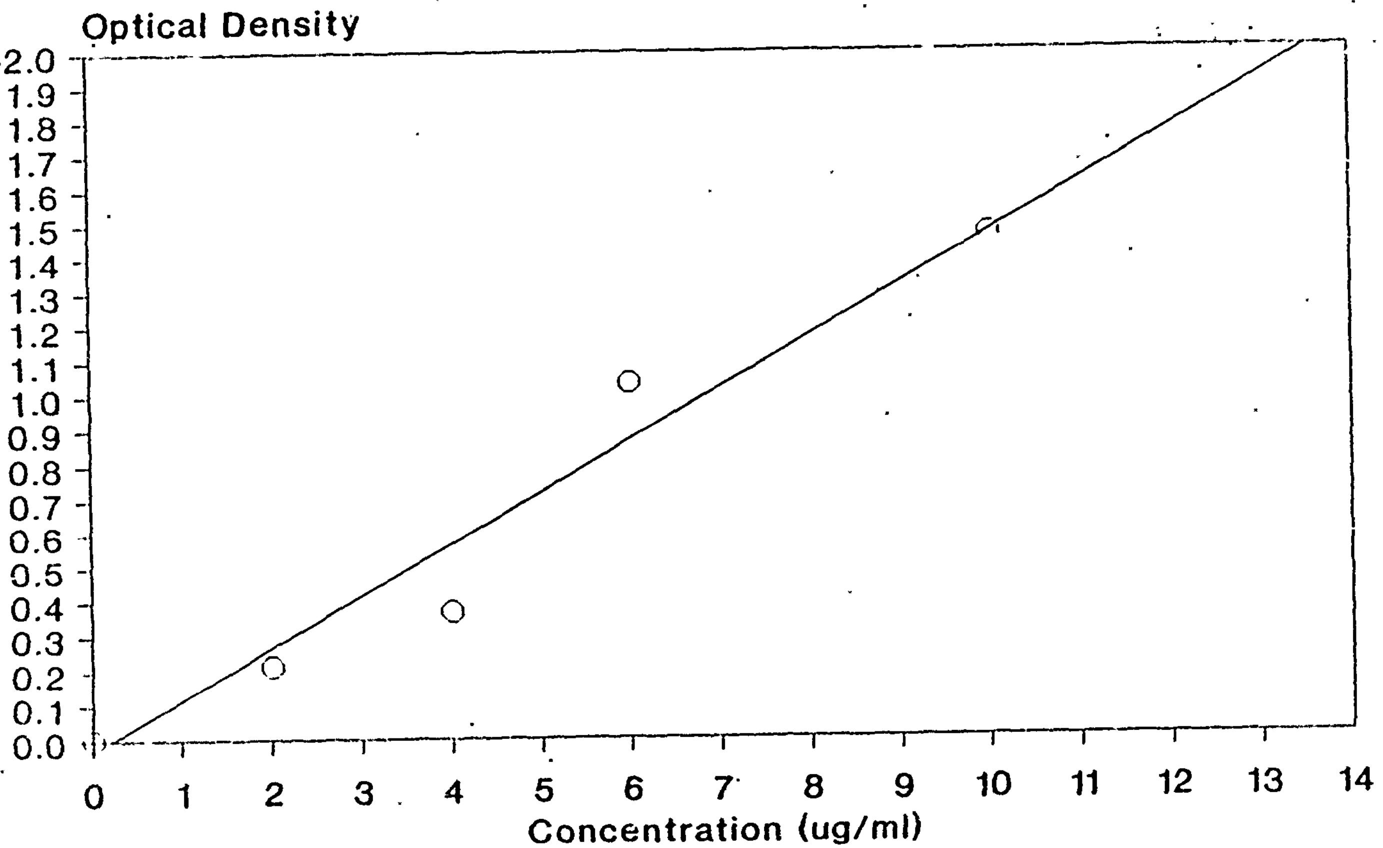
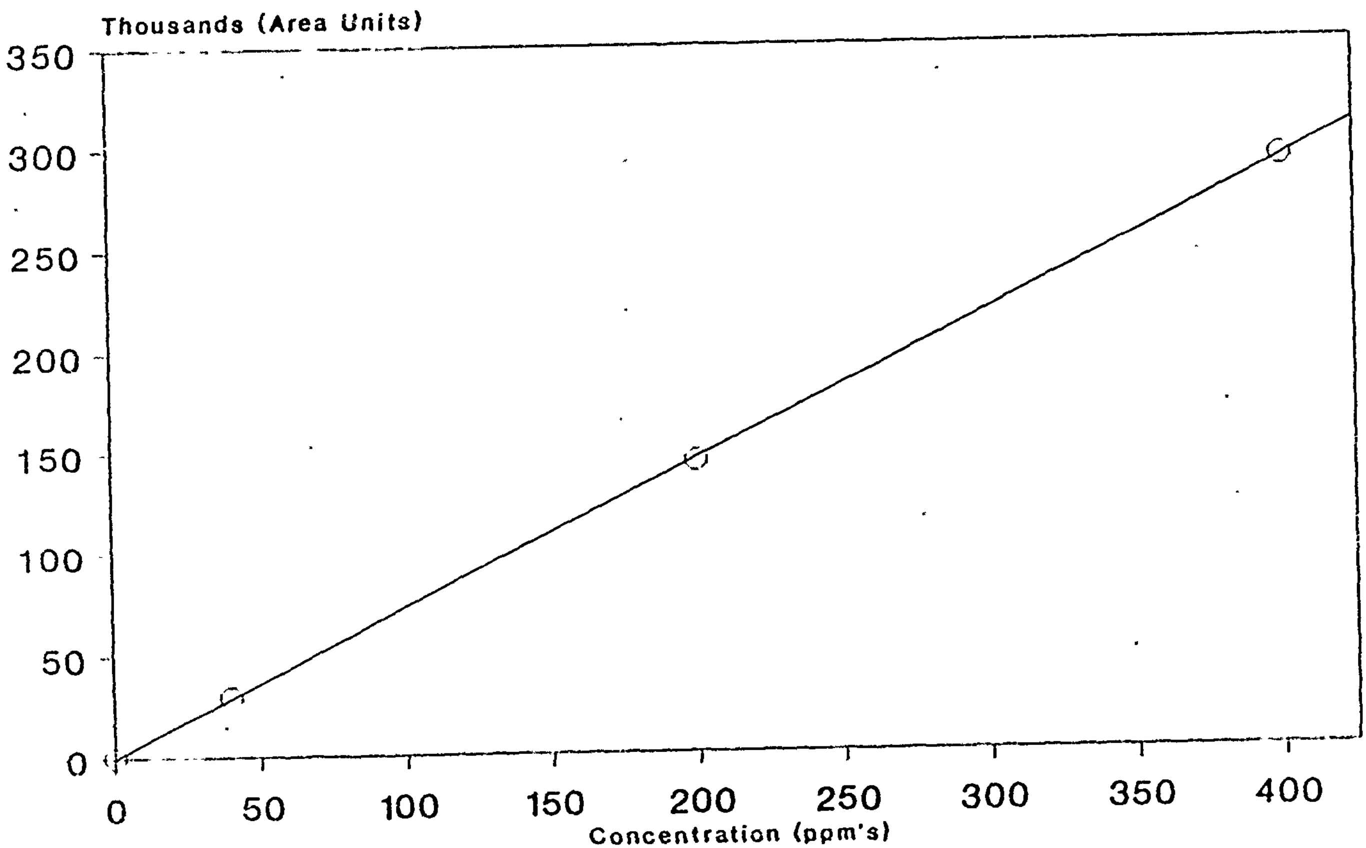


FIGURE 6

Calibration Curve for Residual Acetone Determination by Gas Chromatography(FID) Standards Prepared in Chloroform



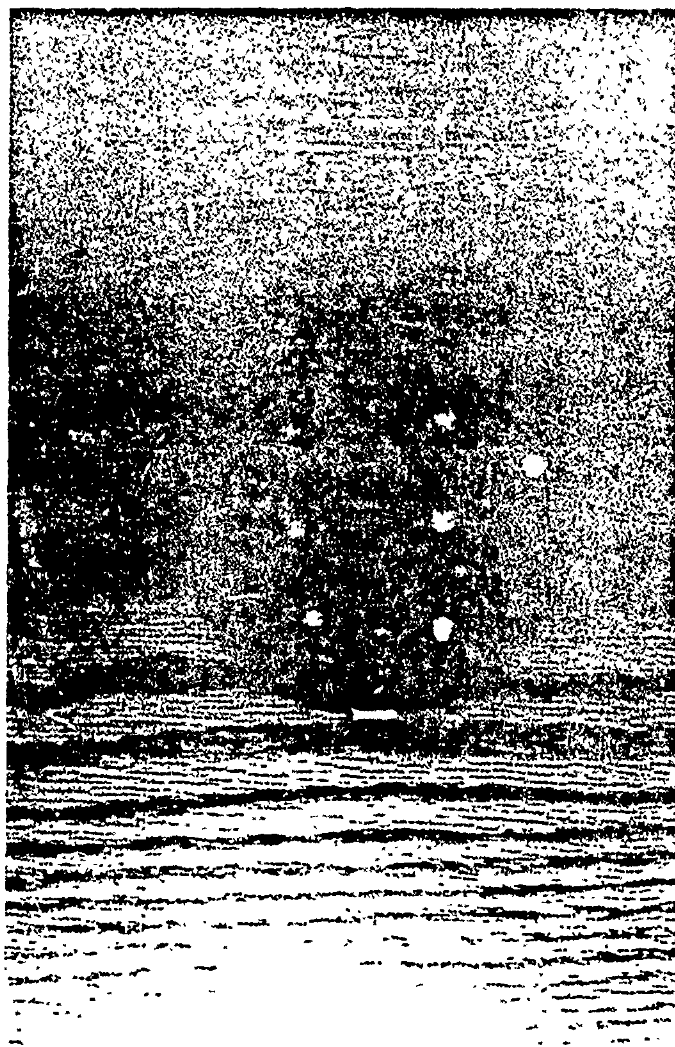


FIGURE 7 - Day 3 Batch MB3 vs. S. aureus ATCC #29996
Dipped 3X in a 10% solution of 65:35 PDLLA-PGA
then spray overcoated with 10% 65:35 PDLLA-PGA
Note placebos on left side.

FIGURE 8

Sieve Analysis of Microspheres
Prepared by Solvent Evaporation
Batches MS1 & MS2

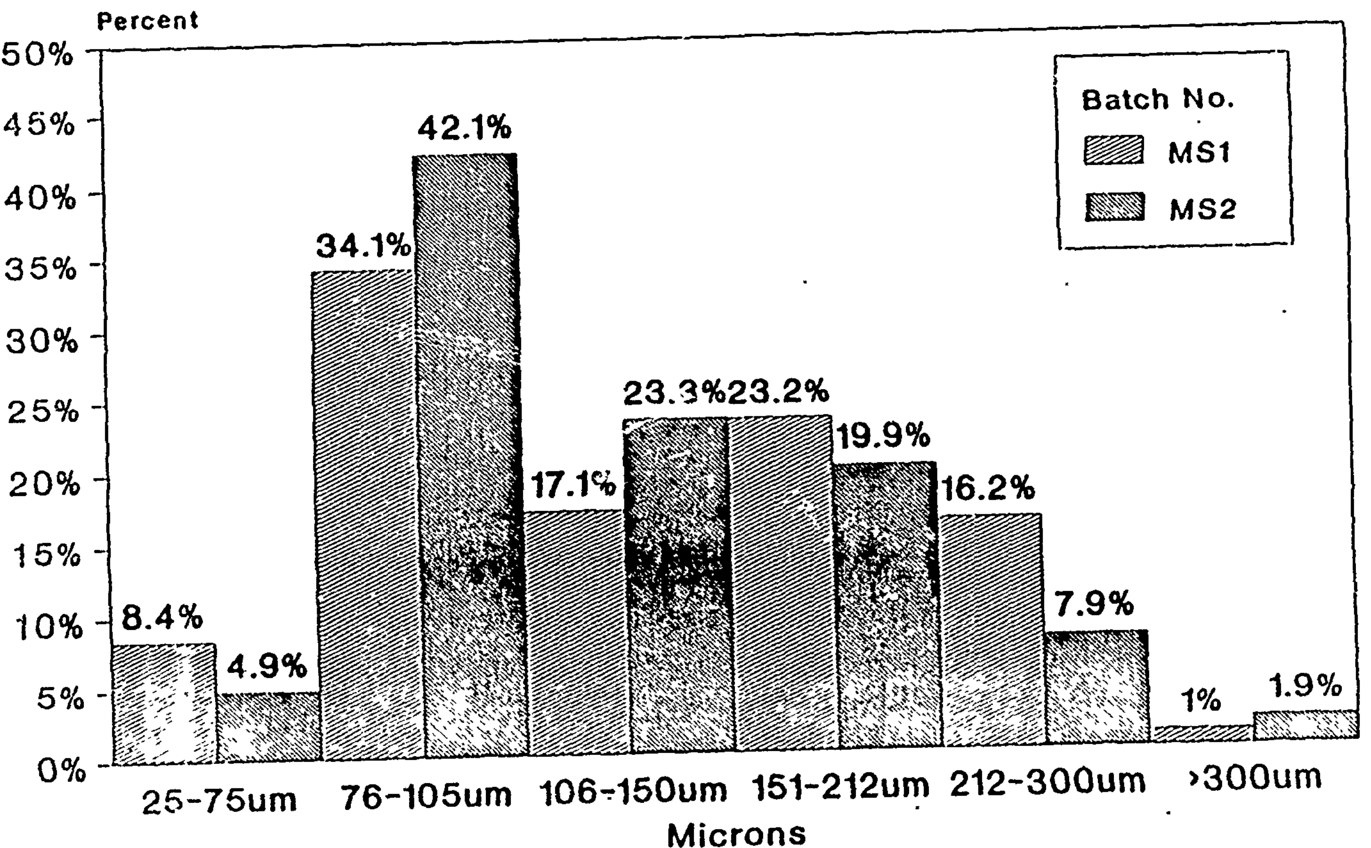


FIGURE 9

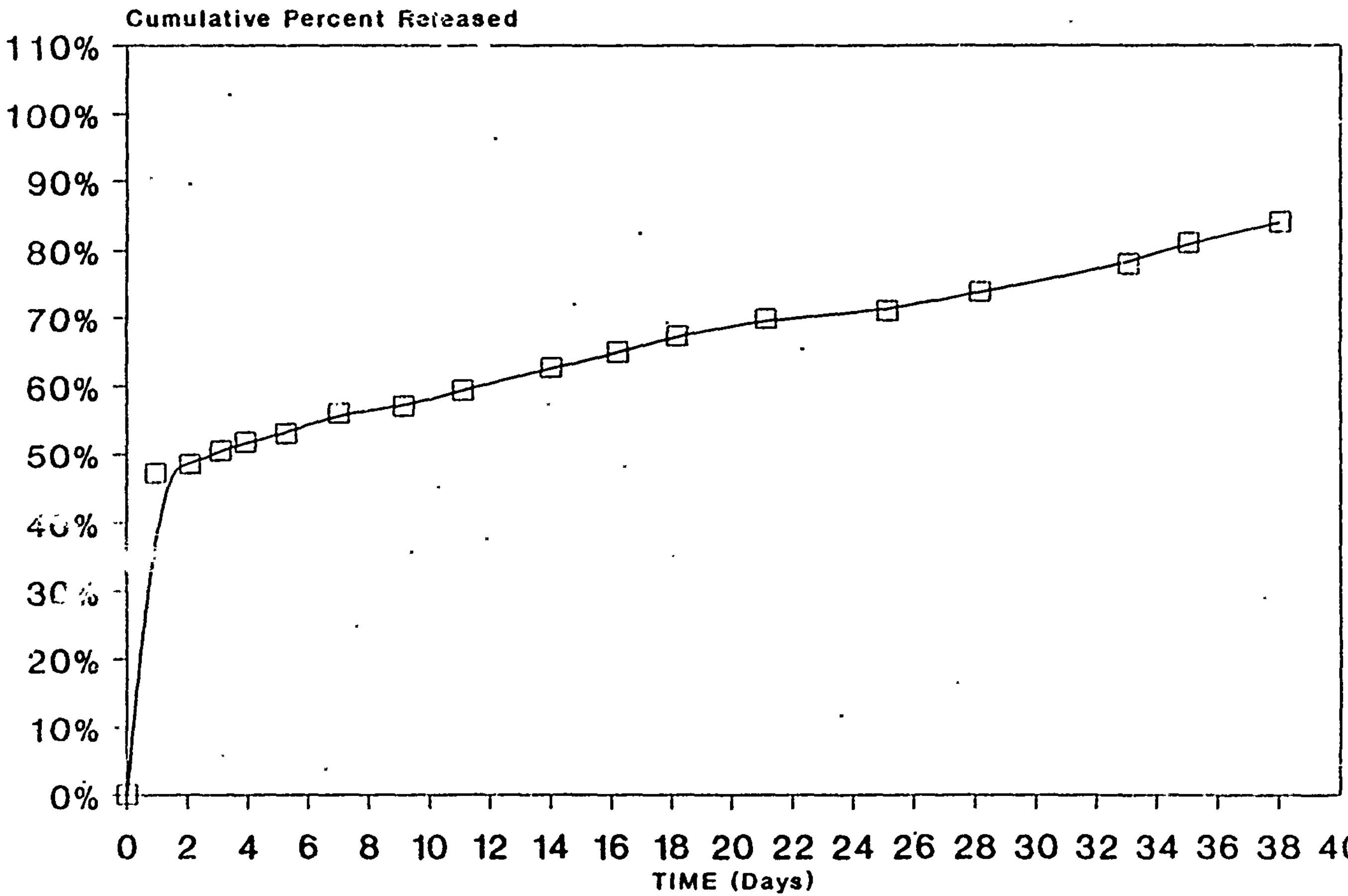
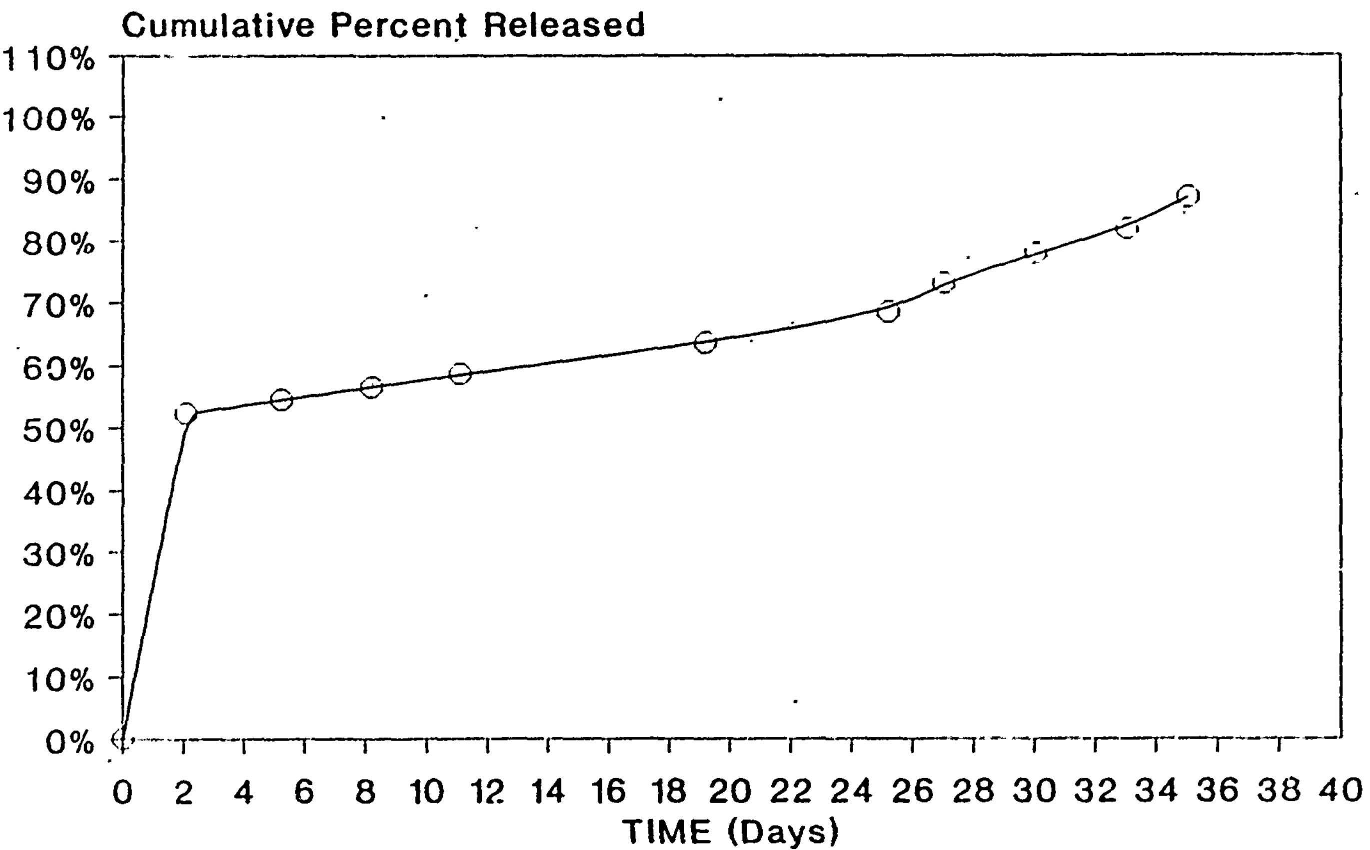


FIGURE 10

In Vitro Release of Tobramycin from Beads - Determination by Reaction w/ o-Phtaldialdehyde @ 333nm



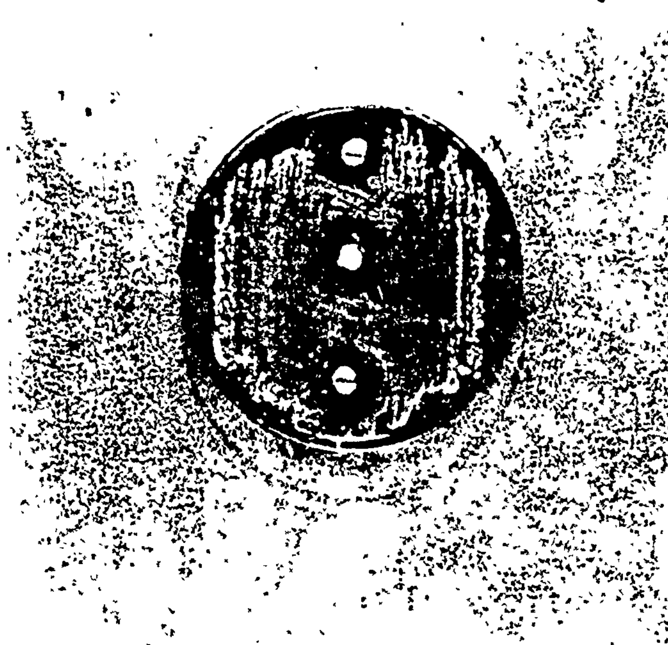


FIGURE 11 - Inhibition of S. aureus ATCC #29996 by Ceftriaxone Beads Batch MB9 after 12 days.

FIGURE 12

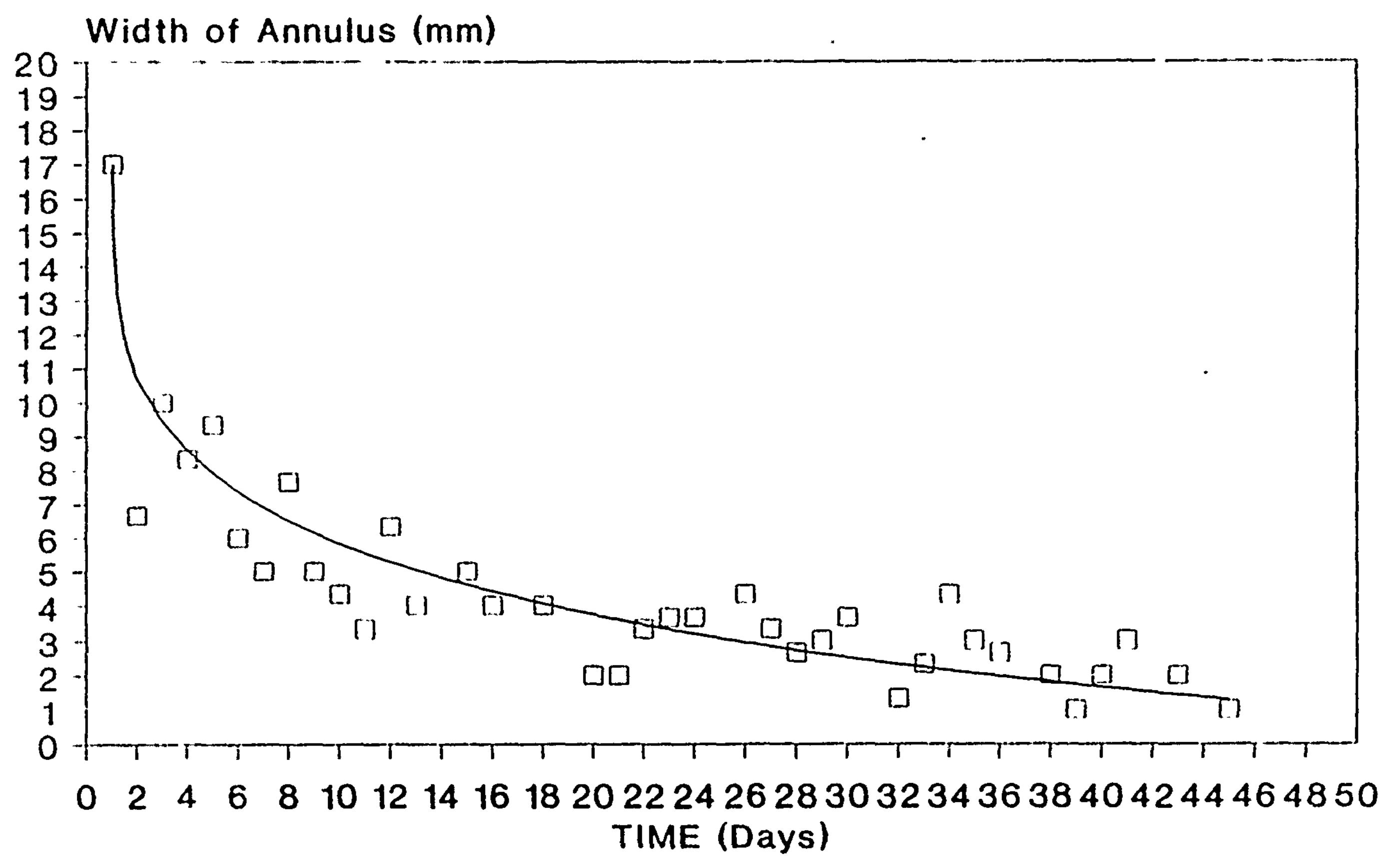
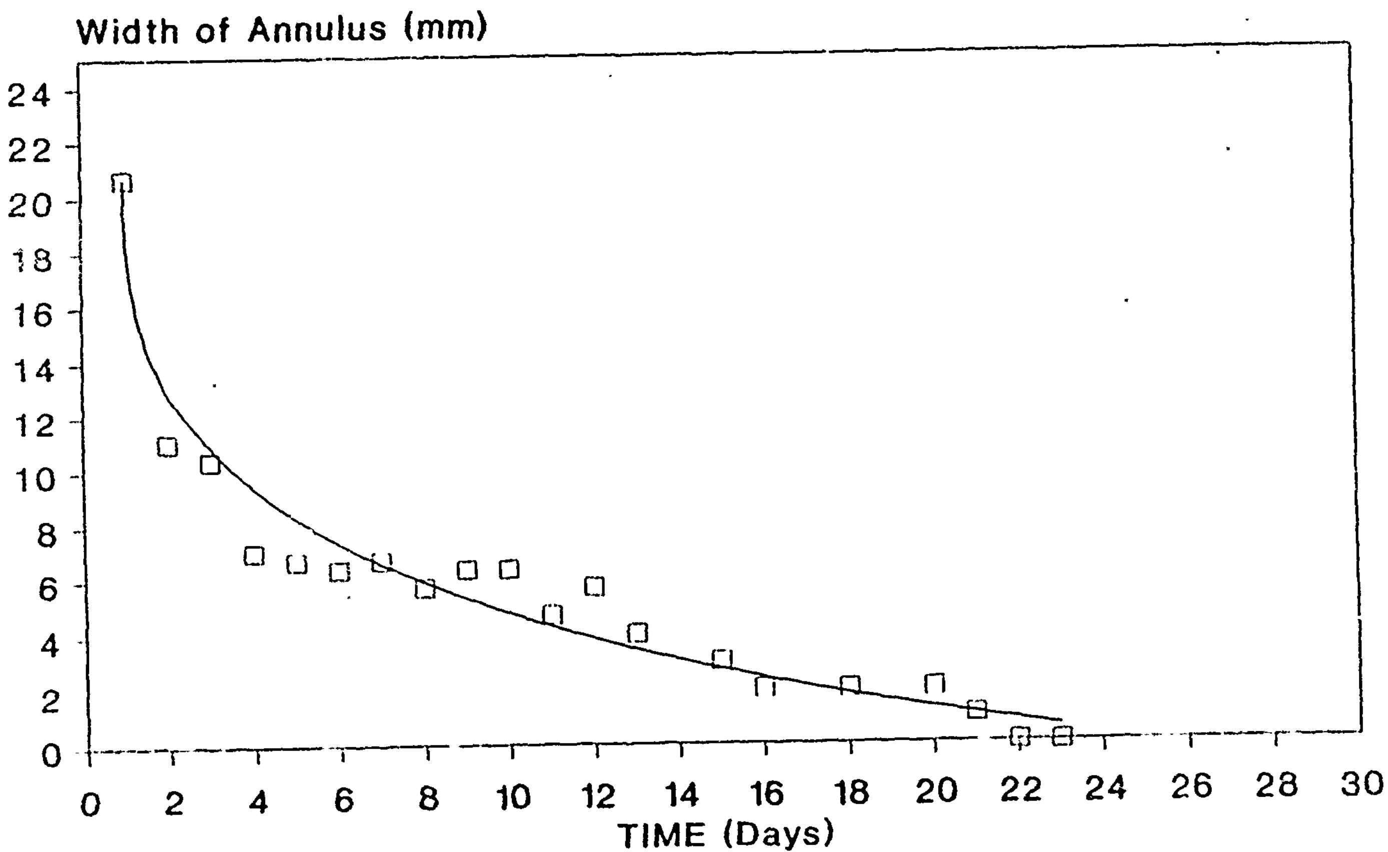


FIGURE 13

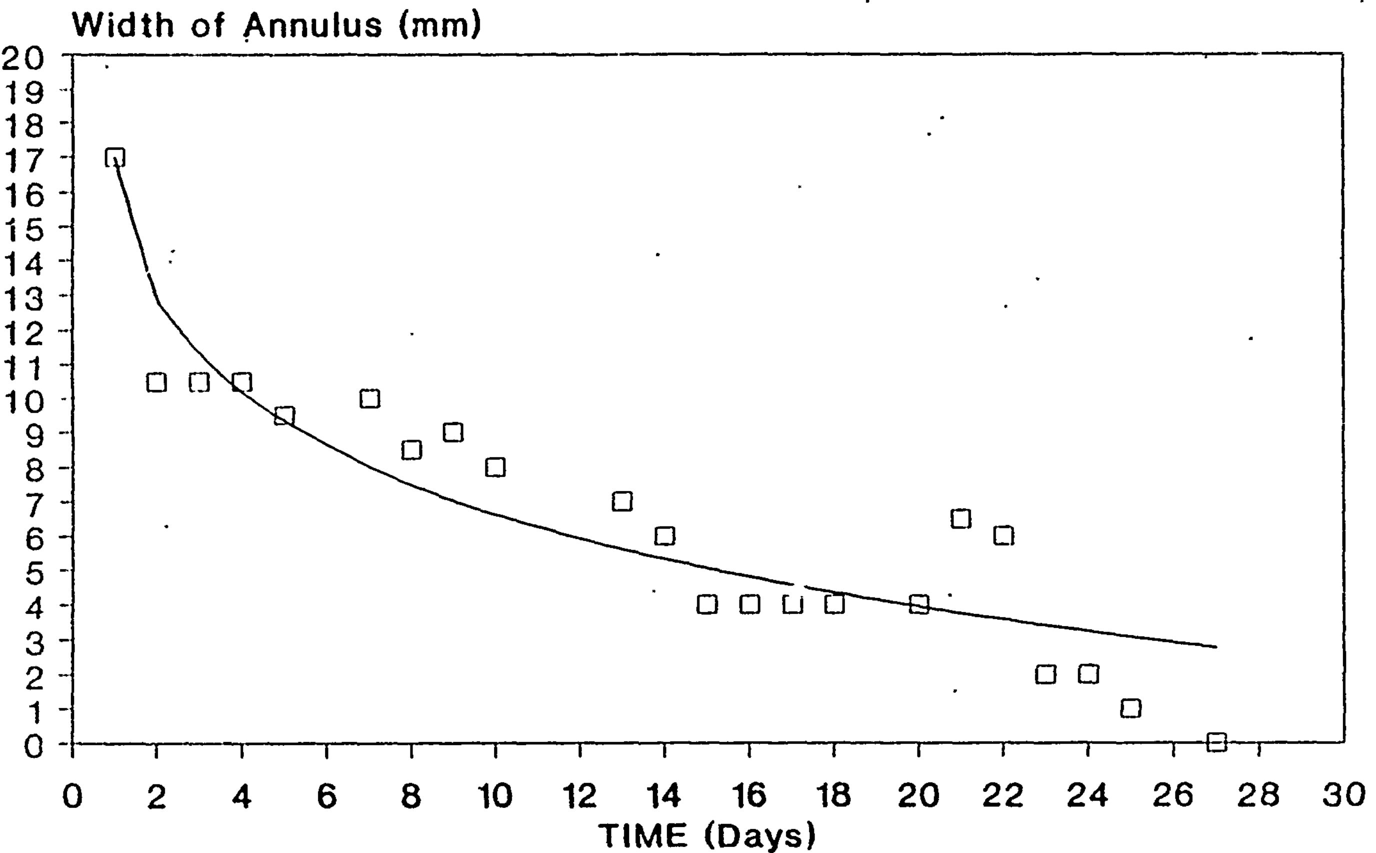
In Vitro Efficacy of Tobramycin Beads
MB10 on Blood Agar Plates Inoculated with P. aeruginosa ATCC 27853



164#18943PAF

FIGURE 14

In Vitro Efficacy of Antibiotic Beads (MB 8) w/ Ceftriaxone and Tobramycin on Agar Streaked w/ S.aureus ATCC# 29996



126#18937MB8

FIGURE 15

In Vitro Efficacy of Antibiotic Beads
(MB 8) w/ Ceftriaxone and Tobramycin on
Agar Streaked w/ P.aeruginosa ATCC 27853