Bioerodible ocular insert for treatment of bovine infectious keratoconjunctivitis in cattle

by

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INTRODUCTION

Statement of the Problem

Bovine infectious keratoconjunctivitis (BIK) is a highly contagious disease affecting cattle. It causes inflammation of the conjunctiva, cornea, and eyelids. It is not fatal, but because of the ocular defects it produces, BIK causes significant economic losses in the cattle industry.

Current treatments of BIK include topical applications of antibiotics or other therapeutic agents using eyedrops, ointments, or sprays, or injections of antibiotics for 5 to 7 days. These methods have shown that any therapeutic agent applied directly to the eye is washed away by lacrimation in few minutes. Repeated applications are necessary to insure recovery. A more efficient method of drug delivery would be of interest.

This study describes the development and evaluation of a ring-shaped device to administer antibiotic to the bovine eye. It is desirable that a controlled-release system could provide a continuous administration of drug to the eye for extended period of time without causing adverse side effects and that it does not require removal from the eye after termination of the therapeutic program (that is, it would

erode in about five days). In this work, the ring device produced is a calcium alginate film with entraped particles of hydrogel containing the antibiotic, tylosin tartrate. The calcium alginate comprizes the bioerodible portion of the device. The in vitro tylosin tartrate release rates for this system are quantified using thin layer chromotography. The erosion characteristics and the drug release rates are discussed in relation to the desired characteristics of a treatment method.

LITERATURE REVIEW

Nature of the Problem

Etiology and nomenclature

BIK is a highly contagious disease affecting cattle of all ages and breed, throughout the world. It is characterized by acute inflammation of the cornea and conjunctiva, accompanied by abundant lacrimation. This disease is known by a number of names including: infectious keratitis, specific ophthalmia, pinkeye, and conjunctivitis. The reddish-pink cast which covers the normal white or pigmented conjunctiva, and the later vascularization of the cornea, undoubtedly lead to the use of the term "pinkeye".

The bacterium, Moraxella bovis (M. bovis), is the main organism that actually causes pinkeye (Jackson, 1953). The bacterium or bacterial colonies can be described, according to Pugh (1969) and Hughes (1981), by the following characteristics:

1. a Gram-negative bipolar staining nonsporulating diplobacillus;
2. hemolytic smooth circular colonies with an entire edge, convex to umbonate, grayish white, and slightly indented into the medium;

3. producing firm easily fragmented colonies which autoagglutinate when placed in most liquid mediums;
4. forming no surface growth in liquid medium but developing a coarse colony which occurs as hemolytic and nonhemolytic types; and
5. proteolytic, oxidase positive, which produces a typical three-zone reaction when grown in liturus milk.

Transmission of M. bovis from one animal to another may be directly by contact with the infectious ocular, nasal and vaginal discharges, or indirectly by flies. It is thought that the M. bovis can survive for 24-72 hours on the surface of the fly. Wind, dust, bright sunlight, and poor nutrition are factors that enhance the likelihood of infection (Baldwin, 1945).

Clinical signs

Important signs of BIK can be summarized as follows (Blogg, 1980):

1. The affected animal will eat less and as a result, in the case of the dairy cattle, will yield less milk;
2. Early in the course of BIK, irritation will be evident by such signs as chemosis, swelling of

the lids, epiphora, bleparospasm, and photophobia;

3. Corneal ulceration and neovascularization accompany this disease, as well as anterior uveitis;
4. A white to deep yellow opacity shows in the corneal stroma in the early days of BIK. With time, the opacity starts to cover the whole cornea. When sight is impaired, range cattle are subjected to many hazards such as drowning, falling, and starving; and
5. After the acute stage, a purulent ocular discharge may be observed.

Economic impact

Pinkeye disease is the fifth most costly disease of cattle in the United States according to the annual losses estimated by the U.S. Department of Agriculture (USDA) in 1976. Both within the United States and world wide, this disease causes significant economic losses. Losses result from diminished growth rate of cattle, loss of body weight of cattle, occasional deaths of cattle, and the expensive cost of treatment. A survey conducted in Australia showed that 81.3% of herds experienced pinkeye within a 5-year period (Slatter et al., 1982). In the United States,

especially in the West, this disease can be a deciding factor between loss and profit.

The following cost estimates are based on literature reviewed by Williams and Gelatt (1981). The USDA estimated that 20% of the 48 million calves born annually in the U.S.A. and 10% of the 30 million feedlot cattle develop pinkeye. It was estimated that the U.S. beef industry lost 160 million dollars in 1976. This figure was derived from an estimate of a loss of $12.96 per infected calf ($120 million), plus treatment costs of $3.00 per treatment for 40% of the infected calves ($12 million). In addition, the average cost of feeding the 3 million infected feedlot cattle for no gain, based on average feeding cost of $0.60 per day for a 14-day no gain period, equals $8.40 per head ($25 million). There is also a $3.00 per head treatment cost for 50% of the affected cattle ($4.5 million). The major cost to the beef industry for the disease for calves was $130 million, while the cost for feedlot cattle was approximately $30 million. This figure included beef cattle only and did not cover the loss of production, weight loss and injuries (including death) in dairy cattle. Dairy cows affected with this disease may exhibit a drop of 25% or more in milk production and become uneconomical. Table 1 was constructed according to the above mentioned estimates

showing a summary of economic losses in the beef industry due to pinkeye in the U.S.A. in 1976 and the U.S.A. and Iowa for 1985 and 1987. The feeder calves include steers and heifers and bulls below 500 pounds, while the feedlot cattle include steers, bulls, milk replacement heifers, and beef replacement heifers above 500 pounds. For 1985 and 1987, estimates were based on a loss of $25 per infected calf, a feeding cost with no gain of $1 per day per infected cattle, and $6 per treatment for each infected calf or cattle. The differences in cost from 1976 to 1985-87 reflected inflation.

TABLE 1. Summary of economic losses due to pinkeye

	1976 USA	1985 IA	1985 USA	1987 IA	1987 USA
			Millions		
FEEDER CALVES					
1. No. of Calves	48	1.50	26	1.20	23
2. No. Developed Pinkeye	10	0.30	5.2	0.24	4.6
3. Infected Calves Losses($)	120	7.50	130	6.00	115
4. Cost of Treatment($)	12	0.72	12	0.58	11
Total($)	130	8.20	140	6.60	130
FEEDLOT CATTLE					
1. No. of Cattle	30	2.50	37	1.90	34
2. No. Developed Pinkeye	3.0	0.25	3.7	0.19	3.4
3. No Gain Feeding Cost($)	25	3.50	52	2.70	48
4. Cost of Treatment($)	4.5	0.75	11	0.57	10
Total($)	30	4.30	63	3.30	58
TOTAL LOSS($)	160	12.5	200	9.9	190

The Bovine Eye

Structure

The sclera covers a major part of the globe. It is very thin at the equator (1 mm), thicker at the corneoscleral junction (1.25-1.5 mm), and at the ceriberiform plate (2 mm) (Severin et al., 1980). It varies among animals, from almost white to a light greenish gray. Much of the coloration is due to a pigmentation of the episclera and the presence of many chromatophores within the sclera. The cornea is elliptical and more curved than sclera. It is highly pigmented around the peripheral area. It is avascular and obtains its oxygen by the aqueous humor, scleral vessels and tears which bathe it. The corneal thickness is important clinically in surgery and disease. The cornea of the bovine is thicker than that of other domestic animals, being 1.5-1.8 mm at the center (Severin et al., 1980). Table 2 lists the corneal and global dimensions in cattle as reported by Martin and Anderson (1981). These data are helpful in anticipating the approximate dimensions that might be needed for an ocular insert.

Tear glands and tarsal glands within the eyelids serve to protect the cornea from drying. The conjunctiva lines the upper and lower eyelids, both sides of the third eyelid, and the surface of the globe. It contains lymphatic tissues

TABLE 2. Average corneal and global diameters in millimeters (Martin and Anderson, 1981)

ANIMAL	CORNEA		GLOBE	
	Vertical	Horizontal	Vertical	Horizontal
Cow	30.42	32.86	40.82	41.90
Calf	23.80	23.14	32.50	34.20

and associated lacrimal glands. The third eyelid, called nictitating membrane, is located nasally and moves over the cornea in a dorsolateral direction. It has a thin pigmented wide free edge with its base being embedded in fat.

The bovine eye has many lacrimal glands that contribute to the secretion of tears. The orbit lacrimal gland, which secretes the serous component to the tear film, is located in the lacrimal fossa of the frontal bone over the dorsal and lateral surfaces of the globe. The nictitating gland contributes a serous secretion and Harder's gland adds a mucoid secretion to the tear film. The ducts which drain the glands are continuous, passing through the puncta to the lacrimal sac. The sac tapers into the nasolacrimal duct which traverses the lacrimal bone and runs upon the lateral nasal wall to drain the tears to the nostril.

Characteristics of tears

The precorneal tear film (PTF) is the fluid covering the normal cornea and conjunctiva. PTF consists of three layers that are approximately seven microinches in total thickness. The outer superficial layer consists of oily materials and phospholipids primarily from the tarsal glands. This layer limits the evaporation of the aqueous layer and provides a binding effect preventing overflow of PTF at the lid margins. The inner mucoid layer is composed of mucoproteins derived from the conjunctival goblet cells. It serves as binding for the aqueous layer to the cornea. The middle aqueous layer consists mainly of water derived from the lacrimal and nictitating glands. Its purpose is to flush foreign materials from the conjunctival sac, to lubricate the passage of lids and nictitating membrane over the cornea, to give a smooth surface to the cornea for optimal optical efficiency, and to provide a medium for transfer of atmospheric oxygen and antibodies to the cornea.

The tear film contains lysozymes which reduce the bacterial count in the conjunctival sac. Tears are a clear, slightly alkaline, salty, watery fluid. The viscosity of tears ranges from 1.053 to 1.405 centipoise. They are isotonic with plasma. The osmotic pressure of human tears is equivalent to that of a sodium chloride solution having a

concentration of 1.014 gm NaCl/100 gm H_2O. This is almost equal to physiological saline (Duke-Elder, 1968). Tears have a pH of 7.4 to 7.5. Water constitutes 98-99% of the tear film; this leads to a specfic gravity close to unity. Tears contain cations such as Na^+, and K^+, and anions such as Cl^-, phosphate and thiocyanates.

Unstimulated flow rates of tears were measured by Slatter and Edwards (1982) by cannulating the nasolachrymal duct and removing the tears with a suction pump. Using 11 animals for 327 collection periods of 30 minutes each, they found a mean tear flow rate of 1.96$\mp$1.84 ml/hr. This is greater than what Hoffman and Spradbrow (1978) reported. Their value of 0.18-1.86 ml/hr was based on two-hour collection periods. This difference could be due to the temperature and duration of the collection periods at which the experiment was performed. Slatter and Edwards kept the cattle housed indoors, in an air-conditioned room at 23^{o}C, while Hoffman and Spradbrow performed their study outdoors. There were large variations in flow rates among cows, but measurements were consistent for individual cows in the Slatter and Edwards study.

Present Treatment Methods

During early stages of an outbreak, affected animals should be removed from the feedlot and isolated. They should be kept in cool, darkened places where fresh water and appetizing food are available. Fly control measures, sanitation, sprays, dusts, as well as systemic insecticides are strongly recommended. Treatment should be applied as early as possible because elimination of M. bovis from a diseased eye will not restore the damaged tissue and will not speed the repair process (Hughes, 1981). Once an infected animal is found in a herd, all animals should be treated.

Early cases of BIK are treated with topical applications of eyedrops, ointments, or sprays containing chemotherapeutic or antibiotic agents. Both eyes of the infected animal should receive treatment. Sprays have retention times similar to eye drops, but sprays cause less irritation. Viscosity-enhancing agents such as methyl cellulose are added to an eyedrop preparation, or the drug is provided as an ointment to sustain intimate drug/eye contact. Injections of various antibiotic solutions and compounds containing chloramphenicol, oxytetracycline, penicillin-streptomycin (Jensen and Mackay, 1979; Blood et al. 1979) or tylosin (Burger, 1970; Hughes, 1981) are also used.

Instilling 3 to 5 drops of 5% silver nitrate solution in the eye, once daily for 3-5 days, is recommended for the severely acute cases of BIK (Jensen and Mackay, 1979). Other antiseptic eye solutions such as 5% boric acid eyewash, 2% mercuro-chrome solution, 5% sulfathiazole ointment, or chloromycetin ointment are used beneficially. Zinc sulfate in a 1:40 solution and ethidium bromide are found to be useful (Hughes, 1981). Both eyes of the infected animal should receive topical treatments with chemotherapeutic or antibiotic agents.

Several methods for topical application of antibiotics have been tried. Sprays containing tylosin tartrate were used twice daily and the clinical signs were eliminated within 5 days (Ellis and Barnes, 1961). Tetramycin eye pellets, which were dropped behind the lower lid, proved to be especially helpful in some outbreaks (Hawley, 1954). The eye pellet consisted of 5 mg of oxytetracycline hydrochloride, 10,000 units of polymycin B sulfate, and 1 mg of tetracaine hydrochloride as the anesthetic. Laboratory data showed a persistance of antibiotic activity in tear samples of 1,295 treated cattle and sheep at a concentration level of 0.67 ug/cm^3 for as long as 31 hours following implantation. In certain cases, some benefit resulted from injections of 0.25 cm^3 of a cortisone solution beneath the

scleral conjunctiva of each affected eye. Sampson and Gregory (1974) showed Tylan® plus neomycin eye powder to be effective in the treatment of BIK when applied once or twice daily for one to three days. Blood et al. (1979) recommended sulphadimidine (100 mg/kg body weight) as a parenteral treatment to provide a therapeutic level in the tears for 12-24 hours. Aronson et al. (1983) recommended daily applications of Tylan® plus neomycin eye powder for a seven day period to treat BIK.

Previous Release Devices

Because a large portion of the drug is washed away by tears immediately after instillation in the eye, repeated applications of drops, sprays, or ointments are needed during a period of a few days. To overcome this problem, Hughes and Pugh (1975) developed and tested a prototype polymer ring. Ring devices were fabricated from polyethylene, polyvinyl, and nylon surgical tubing. Local reaction to the ring device was minimal. Hughes and Pugh reported that retention of a ring in the eye is possible with rings having a circumference smaller than that of the conjuntival sac, but larger than that of the globe. Ring-type devices were retained in the bovine eye for periods up to 19 days. No drug coatings or impregnations were used.

The studies were just restricted to retention of polymer rings and local reaction of the eye to the polymers.

Ocular inserts from soluble and insoluble polymers have been studied and have been found to overcome the difficulty of maintaining a therapeutic concentration of drug at the site of infection. Brightman and Theodorakis (1984) developed an ocular insert comprised of a disc impregnated with chloramphenicol sodium monosuccinate for the treatment of BIK. Dissolved in 100 ml of methylene chloride were 0.8 gm of chloramphenicol sodium monosuccinate, 0.1 gm of tributyl citrate, and 2.0 gm of polylactic acid. After evaporating the solvent, the residue was wrapped in aluminum foil, and then melt-pressed at 170^{o}C for 30 seconds under 3 metric tons load. This produced a film which was cut into oval discs (1.5 mm thick). The disc was attached to the third eyelid of a cow by a spear of biodegradable polylactic acid powder. This insert was retained in the eye for seven days during which the slow release of the drug into the tear fluid provided treatment for the pinkeye. Upon degradation of the spear at the end of seven days, the residual disc was flipped out of the eye by normal blinking. This invention provided a means for controlling both the amount and duration of application of medication. It demonstrated significant advantages such as maintaining a therpeutic

level of the drug in the eye compared with conventional repetitive treatments based on applying ointments and solutions.

Olanoff et al. (1979) developed a tetracycline delivery system made of a trilaminate disc. The core and the coating membranes were fabricated from a series of HEMA (hydroxyethyl methacrylate) and MMA (methyl methacrylate) copolymers. This device demonstrated drug release at constant rates over a prolonged period. The ability of this device to deliver tetracycline at zero-order rates offered unique therapeutic and investigational possibilities.

Weiss et al. (1986) developed and tested nonbioerodible ring devices for treating BIK. The copolymer was based on a 10:90 HEMA:MMA molar feed ratio. Two types of drug delivery systems were fabricated for these studies. Reservoir ring devices were made from copolymer tubing filled with tylosin tartrate powder using a mandrel coating technique. Monolithic ring devices were made by dip-coating teflon, silicone rubber, or polyvinyl chloride tubing in a copolymer/drug solution. The reservoir devices did not exhibit consistent release characteristics. Also, no correlations existed with respect to the wall thickness, mandrel material, and coating method. By comparison, the monolithic devices released considerable amounts of tylosin

tartrate continuously throughout the 9-day test. The in vitro results showed release rate levels above the minimum levels required for treating ocular infections throughout the entire experiment.

Greer and Ryoo (1987) fabricated and tested a tylosin tartrate ocular delivery system for the treatment of pinkeye in cattle. This system was made of a ring shaped polyvinyl chloride (PVC) tubing dip-coated in copolymer/drug solution. The copolymer was based on 10:90 HEMA:MMA molar ratio. Thin layer chromotography was employed to evaluate the release characteristics of this insert. The maximum release rate was observed within the first 8 hours of placing the insert in the elution solution. Release rates were seen to peak in the range of 600 to 700 ug/hr, and then the rates dropped rapidly to approximately 50 ug/hr after 3 days. Results showed that all ring devices released measurable amounts of at least 3 ug/hr of tylosin tartarte for nine days. Eighty to ninety percent of tylosin tartrate was released within the first two days of an in vitro experiment. Inserts were examined under scanning electron microscopy (SEM) to check for surface roughness, cracks, and satisfactory seals at joints. Copolymer/drug coatings on the PVC rings were smooth, except for minor blemishes.

Sustained Release Systems

Recent interest has centered on the idea of replacing the daily administration of a drug with delivery devices that provide a prolonged duration of effective dose to target tissue via a sustained release mechanism. Two types of the membrane moderated sustained release system are: (1) a reservoir device where the drug forms a core which is surrounded by an inert polymer diffusion membrane; and (2) a monolithic or matrix device where the drug is dispersed or dissolved in an inert polymer diffusion membrane network. This section will be devoted to matrix systems since they are of interest in this research.

There are four types of matrix devices. These types can be summarized as follows:

1. The dissolved matrix has the drug dissolved within a crosslinked polymer at or below the saturation solubility of drug in the polymer. The release rate decays according to the time law, $t^{-1/2}$, for the first half of the device life, and then falls exponentially (Baker and Lonsdale, 1974);
2. The dispersed matrix has the drug dispersed as discrete solid particles within a polymer such that the concentration of drug far exceeds its

solubility in the polymer. For a large amount of excess dispersed drug, the release rate falls according to the time law essentially throughout its life;

3. The porous matrix is analogous to the dispersed matrix except that the initial drug load is sufficient to produce contiguous particles of drug throughout the polymer network. The release rate is dependent upon the solvent filled channels produced by leaching of the drug; and
4. The surface treated devices which are analogous to 1-3, but these also have a barrier layer at the surface of the device which is of much lower permeability to the drug than is the bulk polymer matrix.

Matrix devices are useful because they are easily manufactured and because the drug will not be released upon rupture. But there is a potential drawback. The drug release rate will decrease continuously with time. It is evident that the initial release rate per day is very high relative to that seen at long times. This high release rate could be dangerous for a drug with a narrow therapeutic index since toxic levels of the drug could be reached during the early stage of release.

Baker and Lonsdale (1974) have developed equations to describe approximations for drug release rates from dispersed drug monolithic devices. For a cylinder, the fractional release and release rate at any time t, are respectively given by:

$$\left(1 - \frac{M_t}{M_\infty}\right)\left(\ln\left(1 - \frac{M_t}{M_\infty}\right)\right) + \frac{M_t}{M_\infty} = \frac{4C_s Dt}{C_o r_o^2} \qquad (1)$$

$$\frac{dM_t/M_\infty}{dt} = \frac{-4C_s D}{C_o r_o^2 \ln\left(1 - \frac{M_t}{M_\infty}\right)} \qquad (2)$$

where M_t = mass of drug released by time,

M_∞ = total mass of drug,

C_s = solubility in the membrane,

D = permeant diffusion coefficient,

C_o = total concentration of drug, and

r_o = outside radius of the cylinder.

Hydrogels as Ocular Insert Materials

In the 1950s, approaches for incorporating drugs into solid polymers began to be developed and used in agricultural applications (Baker and Lonsdale, 1974). In the 1960s, these approaches were extended to medicine. In the 1970s, new developments showed that large molecular weight drugs (M.W.>600) could be continuously released from solid polymers (Langer and Peppas, 1981).

Hydrogels are polymers prepared by polymerization of appropriate monomers or by crosslinking of already existing polymers. Due to their permeability for low molecular weight solutes, hydrogels have been exploited in designing sustained and controlled release devices. Hydrogels absorb and swell in water, as much as 35% to 95%, without being dissolved (Piskin, 1984). The amount of water absorbed has a great effect on the permeability, mechanical and surface properties, and biocompatibility of the material. Also, the degree of hydration affects the release rate of a drug incorporated within the hydrogel.

Schacht (1984) mentions that solute transport through hydrogels can occur:

1. via a pore mechanism where diffusion occurs through the bulk water retained within pores which exist in the polymer; or
2. via a partition mechanism which involves dissolution of the solute within the polymer followed by diffusion along and between polymer segments.

Nonbioerodible monolithic and reservoir devices can be prepared from hydrophilic and hydrophobic polymers. Hydrophilic polymers, made from monomers such as 2-hydroxyethyl methacrylate (HEMA) (mol.wt. 130.14, see

Figure 1) are water-swollen gels which have poor mechanical properties. Thus their application in the pure state is very limited. The hydrophilic polymers are usually copolymerized with hydrophobic polymers, which are less water soluble, to control the mechanical strength and the degree of swelling. Poly(HEMA) is mostly used in drug delivery systems due to its stability under varying conditions of pH, temperature and tonicity. It is extensively used by the soft contact lens industry because of its biocompatibility and excellent machining and molding properties (Refojo, 1974). It is permeable to body fluids and does not present an impermeable barrier to physiological processes.

Methyl methacrylate (MMA) is a hydrophobic monomer (see Figure 1). When copolymerized with 2-HEMA, the MMA equilibrium water content is reduced below 40%. This has been reported to give a zero-order release of low molecular weight solutes for long periods of time (Cowsar et al., 1976). Poly-MMA is a high optical quality, nonirritating material with excellent machining and molding characteristics. The monomer is moderately toxic when absorbed into the body, so its content in a copolymer should not exceed 0.75% (Estevez and Ridley, 1966).

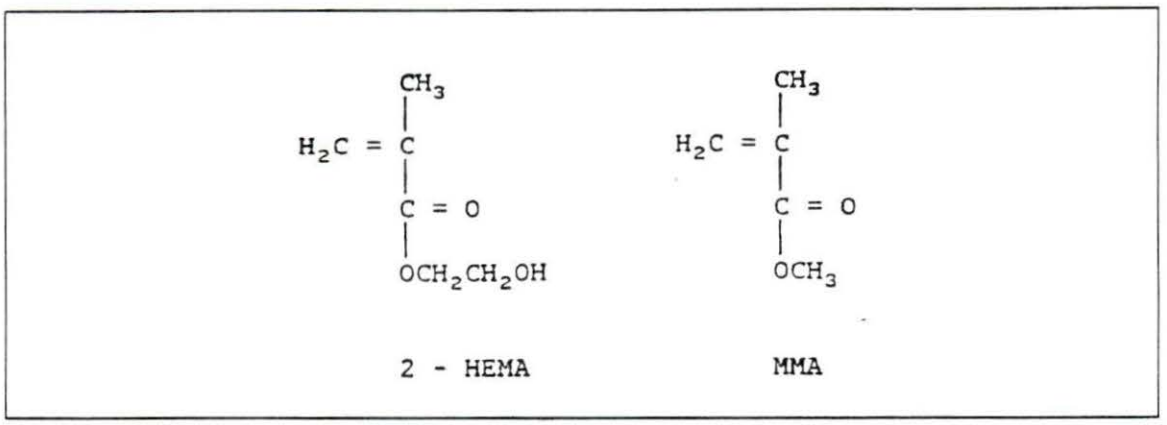

FIGURE 1. Molecular structure of 2-hydroxyethyl methacrylate (2-HEMA) and methyl methacrylate (MMA)(from Langer and Pappas, 1981)

Hydrogels can provide rate-controlling barriers to the diffusion of optimum concentrations of water-soluble drugs to the infected target (Graham and McNeill, 1984). Olanoff et al. (1979) used a 15 mm diameter tetracycline trilaminate system, with a 63:37 MMA/2-HEMA matrix core covered with a 98:2 molar percent MMA/2-HEMA coating of 0.053-0.14 mm thickness, to release tetracycline with a water solubility of 1.7 ug/day. The drug release was found to be a function of the device geometry, disc surface area, coating membrane thickness and composition, and level of core reservoir drug loading. By using different polymeric systems or altering polymer-drug incorporation techniques, a wide variety of release rates were realized.

Alginates as Ocular Insert Materials

Alginic acid is a long chain of poly-saccharides consisting of three main 1,4-linked structural blocks, poly-D-mannuronic acid, poly-L-gulnuronic acid and blocks in which the two uronic acids occur together through 1-4 glycoside linkeage (Badwan et al., 1985). Alginic acid is insoluble in water, but some of its salts are not. Since the polar carboxyl groups are free to react, alginic acid can be changed to an alginate. Alginates of sodium, potassium, and ammonium are soluble in water. Soluble alginates become viscous when mixed with water. When the temperature is decreased, a viscous alginate may be changed to a semi-solid material (gel or jelly). The temperature at which this change occurs is known as the gelation temperature.

If Na-alginate is reacted with a calcium chloride solution:

$$Na_n\ Alg + \left(\frac{n}{2}\right) CaCl_2 \longrightarrow \left(\frac{n}{2}\right) NaCl \quad Ca_{n/2}Alg \qquad (3)$$

where n is an integer. The calcium ion may replace two sodium ions in two adjacent molecules to produce a crosslinking between the two molecules (Skinner, 1973). As the reaction progresses, a crosslinked molecular complex or polymer network forms which constitutes the structure of the gel. Figure 2 shows an example in which some of the sodium

alginate molecules have been crosslinked; some Na^{+} ions have been replaced by Ca^{++} to form calcium alginate.

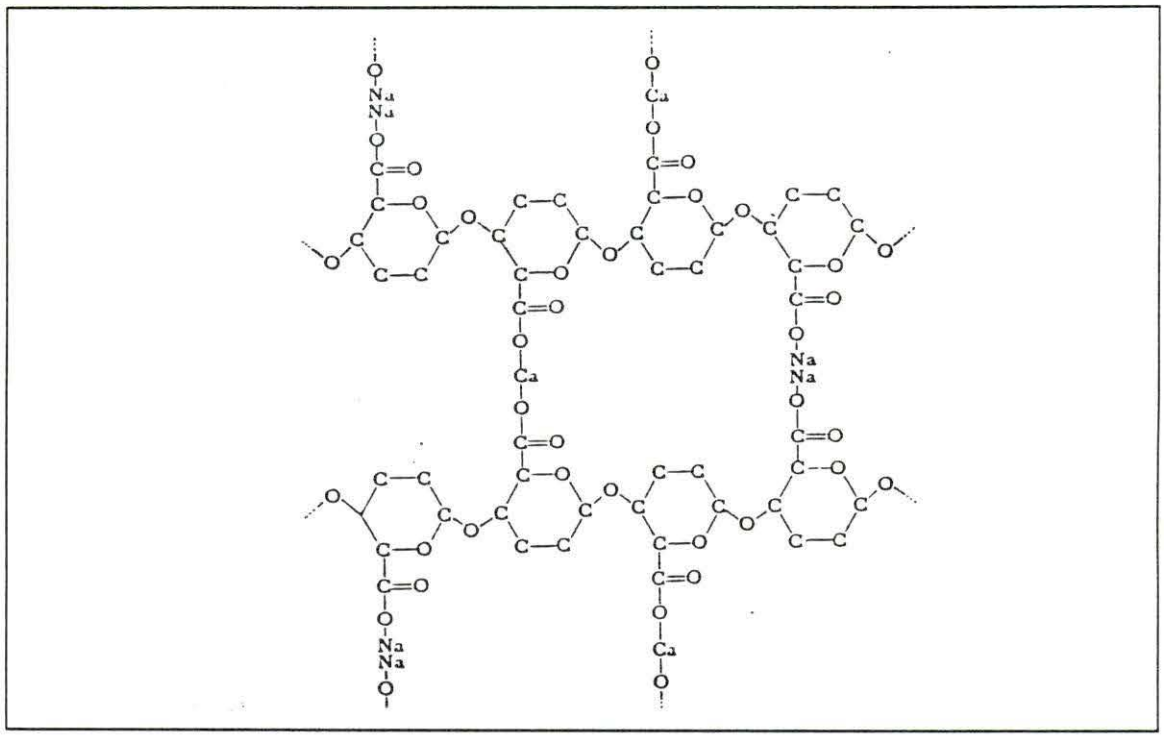

FIGURE 2. Schematic representation of crosslinking of alginate molecules to calcium alginate (from Skinner, 1973)

Crosslinked alginates, such as calcium alginate, are hydrophilic, are compatible with the eye tissues and are bioerodible in the eye (Michaels, 1975). Badwan et al. (1985) prepared and characterized calcium alginate beads impregnated with sulphamethoxazole. They studied the effects of sodium alginate concentration, calcium chloride

concentration and pH, hydration, and compression on the drug release from the beads. Sodium alginate concentration had no significant effect on the release rate. Increasing the calcium chloride concentration and consequently increasing the compactness of the formed matrix, lowered the drug release from the beads. The higher the water content in the calcium alginate matrix the higher the drug release rate observed. This may be attributed to the swelling due to hydration causing cracks which consequently facilitated the sulphamethoxazole release. The drug release from the calcium alginate matrix was through the cracks and fissures in the surface, and a very negligible amount diffused through the matrix body.

Proposed Treatment Method

Selected antibiotic

The proper choice of the level of antibiotic for the treatment of cattle infected with BIK requires a knowledge of the average minimum inhibitory concentration (MIC) for antibiotics against the bacterium *M. bovis* and the distribution of the antibiotic into ocular tissues and tears after subconjunctival administration.

In animal tests, tylosin tartrate has been found to be effective against *M. bovis.* Ellis and Barnes (1961) found

typical colonies of M. bovis in cultures inoculated with samples from 7 of 10 calves which showed corneal opacity and ulceration. Both eyes of the calves were sprayed twice daily for 3 consecutive days with an estimated individual application dose of 30 mg tylosin. The aqueous tylosin tartrate solution was prepared at a concentration of 50 mg/ml. All clinical signs, except corneal opacity and ulceration, disappeared within 5 days, and no reoccurrences were seen. In earlier studies by Pugh et al. (1982), the administration of single application of tylosin, at dosage levels of 5-7 mg/kg, resulted in the elimination of M. bovis from the eyes of 7 of 8 calves.

Tylosin is a macrolide Gram-positive antibiotic isolated from a strain of Streptomycetes fradiae found in soils from Thailand. It is also effective against some Gram-negative bacteria. Tylosin, ($C_{46}H_{77}NO_{17}$) shown in Figure 3, has a molecular weight of 916.14 with 60.30% C, 8.47% H, 1.53% N, and 29.69% O. Aqueous solutions are stable at pH 5.5-7.5 at room temperature for 3 months. It is soluble in organic solvents such as methanol, ethanol, acetone, etc. It is nontoxic and nonirritating to the eye and conjunctival sac. Tylosin tartrate (mol. wt. 1066.2) is commercially available as an agricultural antibiotic. It is soluble in water in amounts greater than 300 mg/ml. It is

stable in aqueous solutions of pH 4-9 at room temperature for at least one month (Ose and Barnes, 1960).

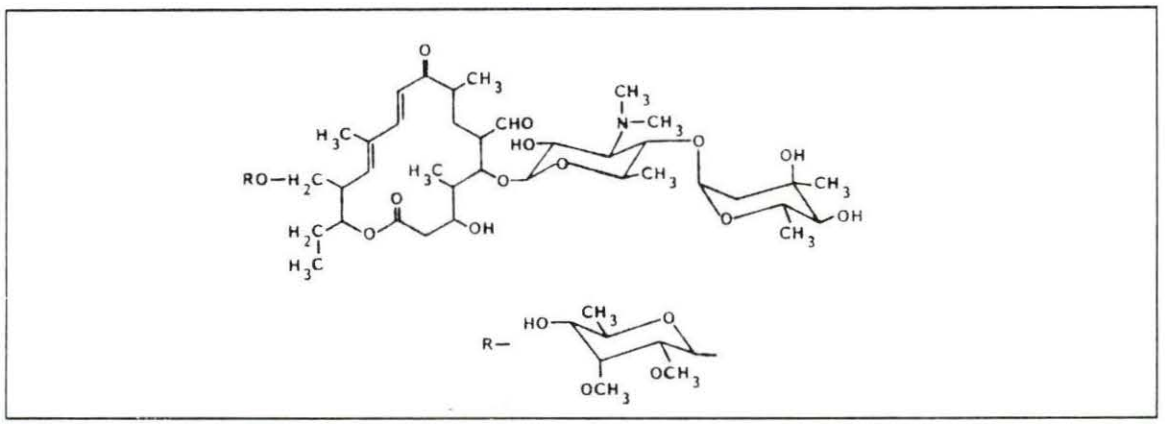

FIGURE 3. Molecular structure of tylosin (from Windholz et al., 1976)

Desirable characteristics of treatment insert

It was decided to attempt and fabricate an insert that had better properties than those previously tried. A ring shaped (Hughes and Pugh, 1975) device that would have a prolonged release of drug, which would maintain a minimum inhibitory concentration of 0.63 ug/ml of tylosin tartrate (Leytem, 1984), and which would undergo erosion after the desired 5-7 days of treatment was the goal. The ocular insert must maintain a minimum tylosin tartrate release rate greater than 1.2 ug/hr (Ryoo, 1986) for the treatment of bovine ocular infections.

To be of practical use, the device must be easy to insert. A ring shape is easy to insert and can remain in the eye for prolonged periods of time without inducing unwanted side effects. Materials selected for use in vivo must be noncarcogenic, nonallergenic, nonthrombogenic, and nonimmunogenic. A copolymer of HEMA/MMA, and calcium alginate can satisfy these conditions; therefore, these materials were used.

PROCEDURES AND MATERIALS

Synthesis of Copolymers and Alginates

The treatment ring devices were fabricated from a copolymer of 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) as the rate controlling membrane, and calcium alginate as the biodegradable matrix.

Preparation of copolymers

Synthesis of the copolymer was by a batchwise process from commercially available monomers as outlined in Olanoff et al. (1979). Based on a feed molar ratio of 10:90 molar percent (m/o) 2-HEMA:MMA monomers, the following materials were added to a one-liter Erlenmyer flask in the order listed: 570.0 ml of absolute ethanol, 380.0 ml of type-one water, 6.1 ml of 2-HEMA,[1] 6.6 ml of MMA,[2] 0.25 gm of sodium persulfate[3] (initiator), and 0.125 gm of potassium persulfate[4] (co-initiator). The flask was placed on a magnetic stirrer, and sealed with a rubber stopper.

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[1]Polyscience Inc., Lot #25295, Ophthalmic Grade, Warrington, Pa.

[2]Fisher Scientific Co., Lot #850960, Fair Lawn, NJ.

[3]Fisher Scientific Co., Lot #745412, Reagent Grade, Fair Lawn, NJ.

[4]Fisher Scientific Co., Lot #852170, Reagent Grade, Fair Lawn, NJ.

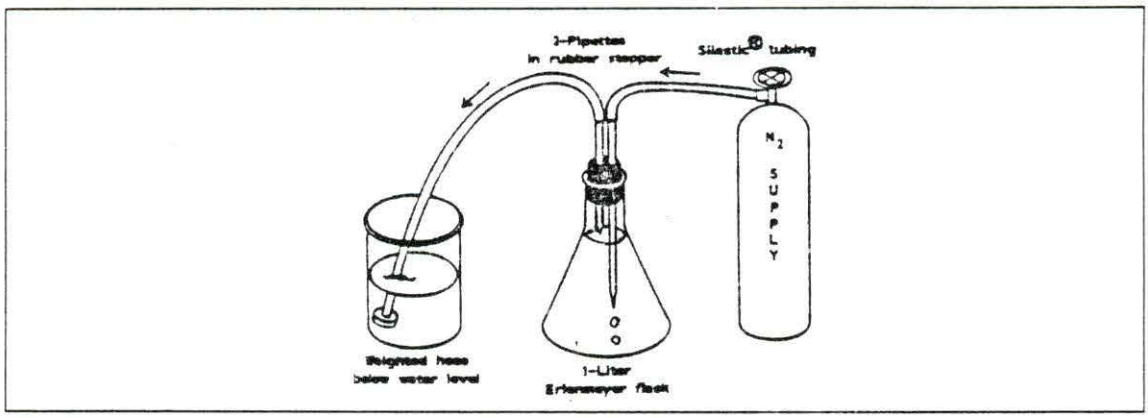

FIGURE 4. Experimental apparatus for copolymer synthesis (from Leytem, 1984)

The liquid contents were purged vigorously with nitrogen for thirty minutes. Then the nitrogen pressure was adjusted to produce a slow continuous bubbling throughout the ten-day copolymerization reaction. This reaction was carried out at room temperature. On the tenth day, the white sticky precipitate which had formed and the ethanol/water solvent were added to three liters of type-one water. The liquid was filtered through Whatman 1-quantitative filter paper in a Buchner funnel by using a low vacuum. Each time the funnel was full, the precipitate was washed four times with 25 ml of type-one water and placed into a Pyrex glass container. The container was covered with filter paper to prevent contamination, and the copolymer was dried in an oven at 50^{o}C for five days under a 25 in Hg vacuum. The

yield of the formed powder copolymer was greater than 90%, and it was stored under nitrogen for future use.

Preparation of alginates

Under vigorous stirring, 7.5 gm of sodium alginate[5] were added to a blender containing 0.03 gm of Tween 80[6] (polyoxyethylene (20) sorbitan monogleate) and 150.0 ml of type-one water. Approximately 12 hours were needed for complete dissociation of sodium alginate. The desired amount of the mixture was placed into a ring mold or spread onto a glass plate. If spread on glass, the material was then dried in an oven at 40°C for 16 hours. The dried sodium alginate was crosslinked with different polyvalent metal cations (specific cases selected within an atomic number range of 13 to 56) to form nontoxic alginates (Michaels, 1975). Portions of the sodium alginate film were immersed in a 5.5 weight percent (w/o) zinc chloride solution (pH=4.5) for 5 hours, a 10.0 w/o aluminum chloride solution (pH=3.1) for 5 hours, a 30.9 w/o barium chloride solution (pH=4.5) for 12 hours, a 30.0 w/o calcium chloride solution (pH=6.7) for 12 hours, a 20.0 w/o ferric chloride solution (pH=4.0) for 12 hours, or a 20.0 w/o cadmium

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[5]SIGMA Chemical Co., Lot #43F-0839, Alginic Acid(VI), St. Louis, MO.

[6]Fisher Scientific Co., Lot #752139, Fair Lawn, NJ.

nitrate solution (pH=4.0) for 12 hours. Three more calcium alginate films were prepared at three different crosslinking times of 12, 24, and 41 hours. The crosslinked alginates were dipped twice into a 50 w/o glycerine bath and dried at room temperature. They were stored in a dessicator for bioerosion studies.

Production of The Treatment Device

Testing of degradation of alginates

Three samples, 7 to 11 mg each, of each of the crosslinked alginates were shaken in 10 ml of 0.9 w/o sodium chloride solution (saline) at 37^{o}C to test for degradation. Every hour, the sample was removed, dried between two filter papers with 100 gm load on top, and then weighed. The weight of the dry sample was compared with its original weight to determine degradation by obtaining the weight loss of the sample. The sample was put in fresh saline and returned to the shaking bath. Data were collected for seven consecutive days. The samples were not reshaken overnight.

Fabrication of the mold

The insert was fabricated in a ring-shaped mold. The sodium alginate mixture was poured into a mold which had a central movable part. While drying in the oven, the alginate film shrank and tightened around the central

portion. The central part was made movable so it could be pushed down to free the tight alginate ring. The movable part was screwed to a teflon stationary portion to ensure a proper diameter and thickness for the alginate ring. The outer part of the mold was made of aluminum to prevent the teflon from distorting when heated in the oven. The mold had a 2 cm^3 volume capacity; it had a 43.0 mm outside diameter, a 32.8 mm inside diameter, and a 3.2 mm depth.

Preparation of copolymer/drug particles

Twenty milliliters of dimethyl formamide[7] were mixed with 1 gm of copolymer powder and heated to 50°C for 6 hours for complete dissociation of the copolymer. After cooling the mixture to room temperature, 500 mg of tylosin tartrate[8] were mixed into the solution and stirred continuously overnight. The final copolymer/drug mixture was yellow and viscous. The mixture was dried in the hood for 5 days at room temperature and then transferred to a dessicator containing $CaSO_4$ (Drierite). Liquid nitrogen was mixed with the copolymer/drug precipitate which was then crushed in a ceramic crucible to 700-1000 um particle size.

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[7]Fisher Scientific Co., Lot #856540, Certified, Fair Lawn, NJ.

[8]SIGMA Chemical Co., Lot #124F-0195, St. Louis, MO.

Production of the bioerodible insert

One and half milliliters of sodium alginate mixture was added to the ring-shaped mold, and dried in a 40°C oven for 4 hours. Then 150 mg of the crushed copolymer/drug particles were dispersed over the sodium alginate film. The particles were covered with a layer of sodium alginate mixture and dried in a 40°C oven for 4 hours. The final ring device was immersed in a 30 w/o calcium chloride solution (pH=6.7) for 12 hours. The sodium alginate reacted with the calcium chloride solution producing crosslinking between two molecules. The calcium crosslinked alginate insert was dipped twice in a 50 w/o glycerine solution and stored in a nitrogen hood for drug release studies.

In vitro drug release experiment

Figure 5 shows a drug elution system used to test ring devices for drug release rates. Each ring was placed in the inner ring-shaped hole. This system was placed in a water bath maintained at 37°C. The elution solution in the reservoir consisted of a mammalian Ringer's solution (8.60 g NaCl, 0.30 g KCl, and 0.33 g $CaCl_2$ in 1.0 liter of distilled water). The flow rate was adjusted to approximately 2.0 ml/hr as recommended by Ryoo (1986). Samples were collected in vials for 1 hour periods, except for overnight collections which were collected for about 10 consecutive hours.

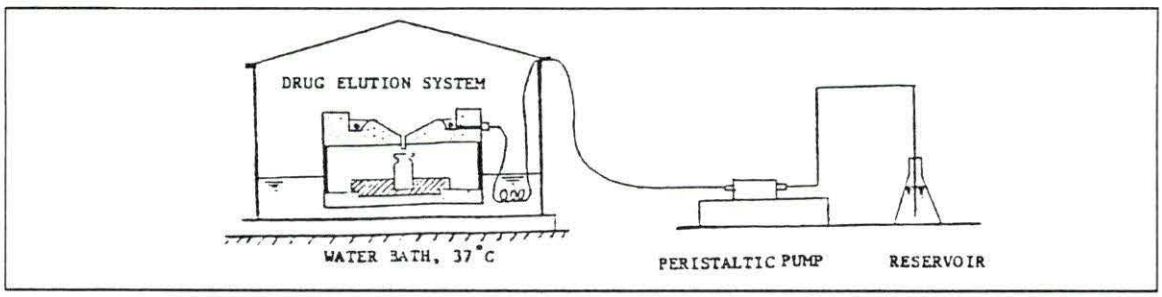

FIGURE 5. Drug elution apparatus (from Ryoo, 1986)

TLC quantitative analysis of tylosin tartrate

The amount of tylosin tartrate in the collected samples was determined using thin layer chromotography (TLC). This is a separation method in which a uniform thin layer (200 um thick) of silica gel serves as the stationary phase. Whatman LKC18F, 20x20 cm, TLC plates[9] were fully developed in a glass chamber containing 85 volume percent (v/o) methanol and 15 v/o distilled water solution (the mobile phase). The developed plates were air-dried at room temperature for 3 days.

Collected samples were dried in a 50°C oven, then diluted in a volume of water equal to one tenth the original sample volume. Since the salts (NaCl, KCl, and $CaCl_2$) in the sample solution could affect the spot size and the R_f value on TLC plate, the standard drug solutions were made

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[9]Whatman Chemical Separation, Inc., Clifton, NJ.

with a concentration of salts to approximately match the redissolved samples (Leytem, 1984). R_f is defined as the ratio of center-of-sample distance from zero reference to the developing-solvent-front distance from the zero reference. The preadsorbent layer-stationary phase interface is the zero order reference. Therefore, four tylosin tartrate standards of known concentrations (4.0, 2.0, 1.0, and 0.5 ug/2.5 ul) were made with a salt concentration equal to ten times that in the original release medium (Leytem, 1984). Two and half microliters of each drug standard and diluted sample were applied onto a TLC plate 1.5 cm below the preadsorbent layer line using a 0-10 ul pipett.[10] When the spots were dried, the plate was developed in the methanol solution to a level 6 cm above the preadsorbent layer line. The developed plate was dried at room temperature, and the spots were visualized using an ultraviolet light source[11] to give a preliminary indication of their intensity. If spotted samples were darker than all the drug standards, new samples were prepared by dilution and were then spotted on another plate, developed, visualized, and compared with the standards. Direct visualization of the spots was done by spraying the

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[10]Drummond Scientific Co., Broomal, PA.

[11]UVP, Inc., Model UVGL-25 Mineralight® , San Gabriel, CA.

developed area of the plate with 10 v/o sulfuric acid and 90 w/o methanol solution at a rate of 15 ml/min for 15 seconds. The sprayed plate was placed in a 100°C oven for 5 minutes, and then taken to a Knotes fiber optic scanner[12] for scanning within the next hour. The densitometer measures the spot intensity by a method of cross-scanning the TLC plate (perpendicular to the direction of development). Settings used were as follows: 10 cm/min scanning speed, 1 or 2 attenuation, B-A scanning mode, and normal output. The phosphorous coated disc had a 615 emission peak, with a 10 nm bandwidth. The plate was placed on the tracks with the gel side up. The densitometer was connected to an IBM AT[13] personal computer through a Keithley[14] A/D convertor (System 570). The data were saved on a disc. The BASIC computer program used Soft500[15] (a software package for data aquisition).

After transferring the data to the disc, peak curves were displayed on the computer screen. The area under each peak was calculated. This corresponded to the concentration of tylosin tartrate in that spot.

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[12]Knotes Scientific Instrument Group, Model 800, Vineland, NJ.

[13]IBM Corporation, Boca Raton, FL.

[14]Keithley Data Acquisition and Control, Cleveland, OH.

[15]Keithley Data Acquisition and Control, Cleveland, OH.

RESULTS

Bioerosion of Crosslinked Alginates

Samples of the crosslinked alginates were tested for their bioerosion rate in a saline bath. Appendix A summarizes the daily weights of the tested samples. Results showed that sodium alginate is totally soluble in saline within a few minutes. Zinc and cadimium alginates, crosslinked for 12 hours, dissociated partially in saline after 1 day. They became tacky and hard to handle. Barium, iron, and aluminum alginates,crosslinked for 12 hours, lost 20-29% of their original weight after 1 day. Those alginates showed minor weight losses for the rest of the erosion experiment. On the other hand, calcium alginates, crosslinked for 12 hours, lost 24% of their original weight after 1 day, and became tacky and easily broken after 3 days. Swelling of these samples was observed. Calcium alginates dissociated completely after 5 days. The bioerosion test was repeated for the calcium alginate crosslinked for 12 hours. Similar curves for erosion rates of calcium alginate were observed (Figure 6)

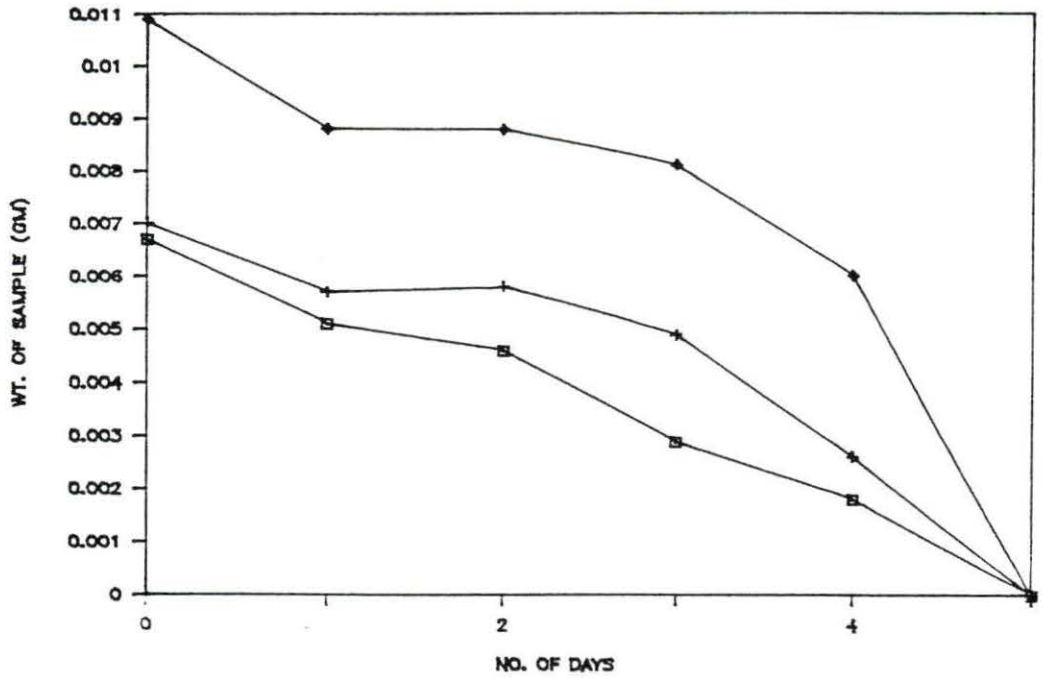

FIGURE 6. Calcium crosslinked alginate degradation (12 hours crosslinking)

Increasing the crosslinkling time of sodium alginate in the calcium chloride solution resulted in strengthening the calcium alginate film and therefore decreasing its bioerosion rate. Figure 7 shows calcium alginate degradation for 3 different crosslinking times. For a crosslinking time of 12 hours, the calcium alginate dissolves completely in about 7 days. By comparison, for crosslinking times of 24 and 41 hours, the calcium alginate dissociates completely in about 8 days.

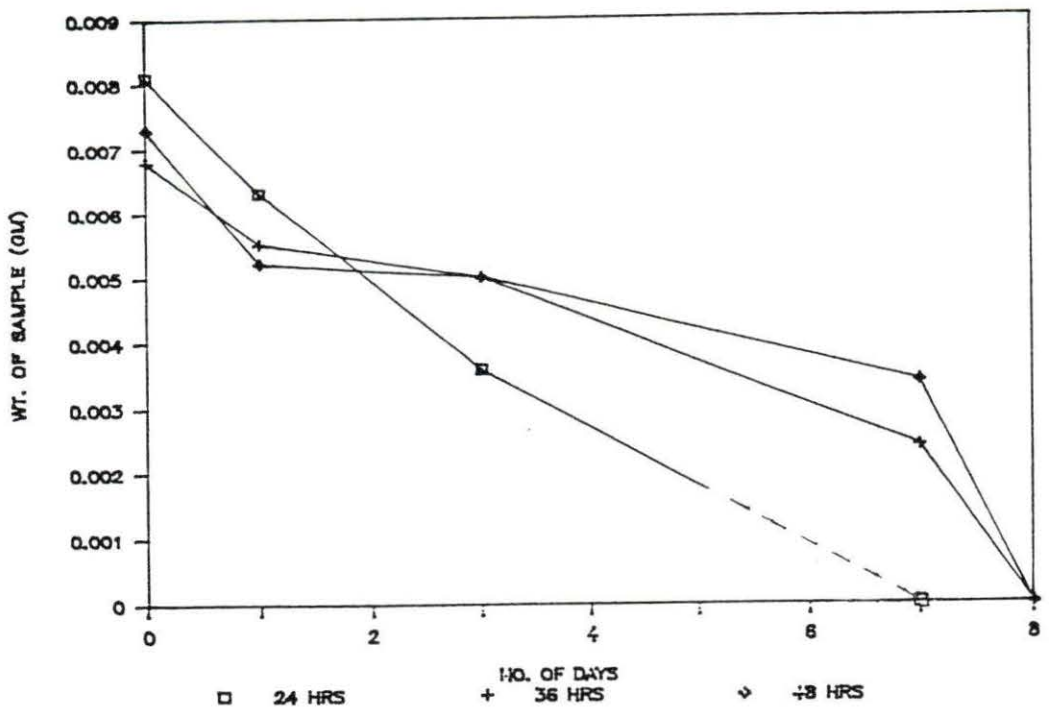

FIGURE 7. Calcium crosslinked alginate degradation for different crosslinking times (in hours). The sample for the lower curve for days 3 to 7 was seen to be firm through day 6, and seen to dissolve completely by day 7.

Tylosin Tartrate Release Characteristics

Copolymer/drug particles

The copolymer/drug precipitate was crushed into small particles and dispersed in a ring-shaped Whatman filter paper (Ashless 4). The purpose of this experiment was to evaluate tylosin tartrate release characteristics from the copolymer. After the release experiment, samples were

dried, diluted with distilled water, and spotted on TLC plates to determine the drug release rate for each sampling period. Quantitative TLC analysis of tylosin tartarte from the hydrogel included: collection volume, release time, dilution volume, amount of drug in the spot, total drug in the sample, release rate per hour, and cumulative drug release.

Appendix B contains the data for drug release from the copolymer. The tylosin tartrate release rate and the cumulative tylosin tartrate released are plotted versus the release time in Figure 8. The cumulative drug released increases with the release time throughout the experiment period. The initial drug release rate is high, reaching a maximum of 1154 ug/hr after 8 hours of the release test. Then the release rate decreases exponentially reaching a minimum of 12 ug/hr after 3 days, where it remains throughout the rest of the test period. After 5 days, the release rate ranges from 15 to 29 ug/hr of drug. The ratio of cumulative tylosin tartrate released to the tylosin tartrate loading is about 41 w/o (Table 3) for 5 days.

The bioerodible insert

The crushed copolymer/drug particles were entraped in a calcium crosslinked alginate matrix which comprized the bioerodible part of the insert. Quantitative TLC analysis

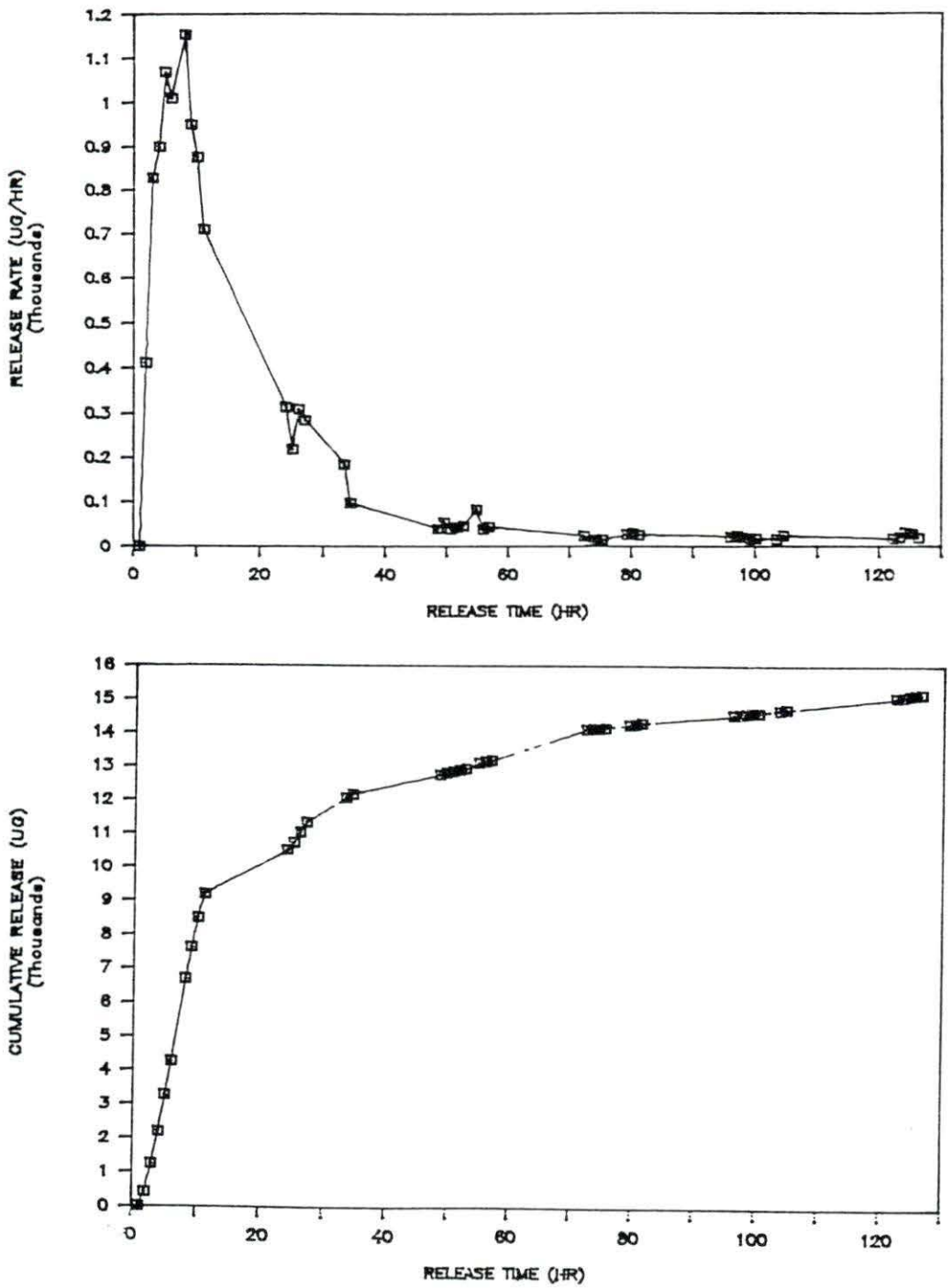

FIGURE 8. Release characteristics from copolymer H4

TABLE 3. Comparison of release characteristics

Device No.	Initial Release Rate (during 2 days) ug/hr	Release Rate Level (after 5 days) ug/hr	Total Release Amount (for 5 days) ug	Ratio of Release Amount %
H4	42-1154	15-29	15146	30.3
D3	17-510	6-10	6333	12.7
D5	34-672	9-27	12154	24.3
Ryoo (1986)				
Group I	77-657	7-50	15275-21886	30.9-42.3
Group IV	62-925	3-22	15851-18273	28.7-36.8

was employed to evaluate tylosin tartrate release from the bioerodible insert.

Figure 9 shows a computer printout of a scanned plate from device D3. The printout shows the area under each peak, the number of drug standards and samples, and the concentration of tylosin tartrate in each of the collected samples. "s" and "t" refer to Simpson's rule and trapezoidal rule. Simpson's rule was chosen for curve area evaluation for this analysis.

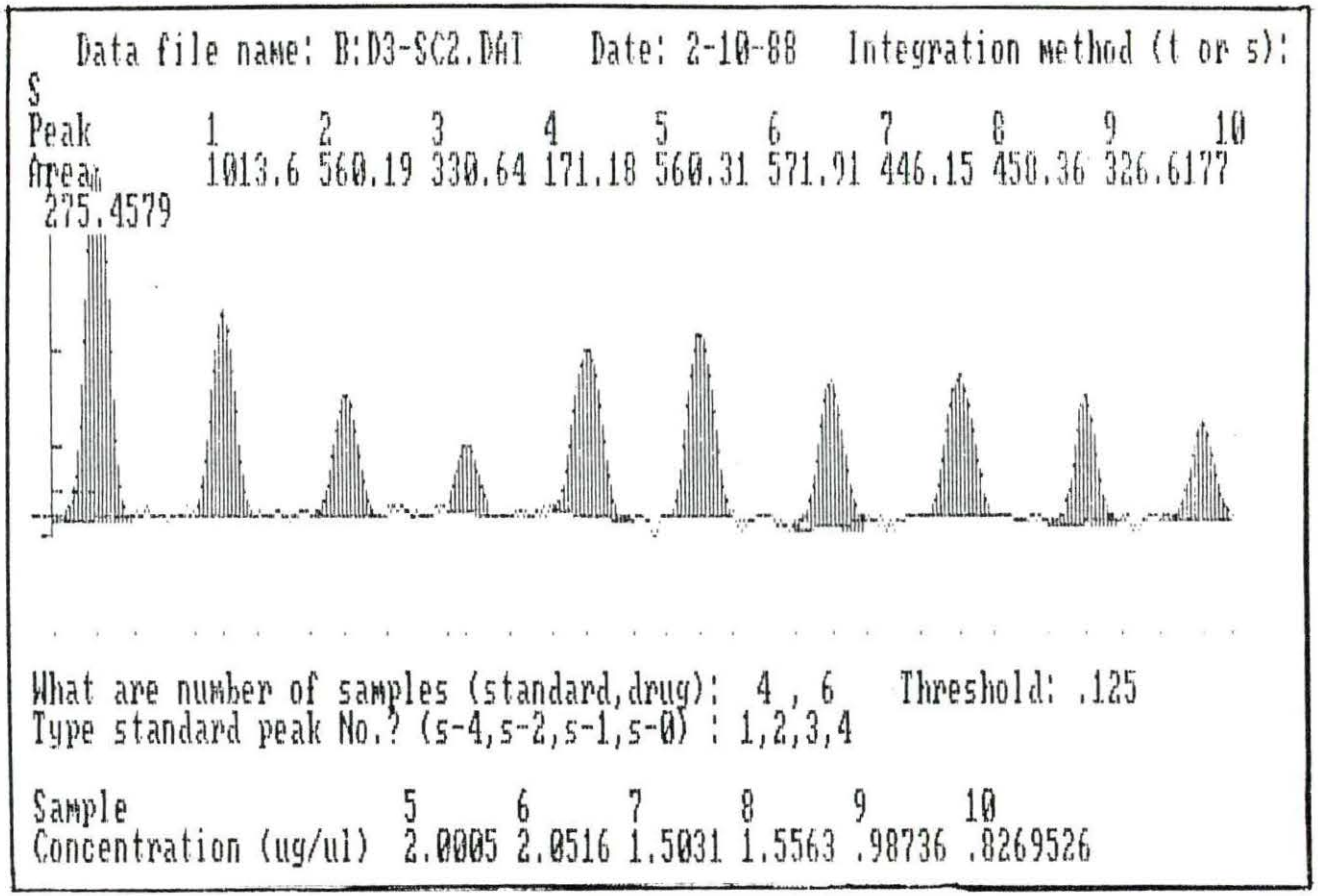

FIGURE 9. Computer printout of a scanned plate

Appendices C and D contain data on release from devices D3 and D5. Figure 10 shows the tylosin tartrate release curves for device D5. The initial drug release rate reaches a maximum of 672 ug/hr after 4 hours. After 5 days, the release rate reaches a minimum of 9 ug/hr of drug. The drug release rate drops exponentially with time. Measurable amounts of tylosin tartrate (9-27 ug/hr) are detected after 5 days. The ratio of release amount is 39 w/o (Table 3) for 5 days. The cumulative drug released increases with time throughout the test period (Figure 10).

Similar results are observed for drug release device D3 (Figure 11). The initial release rate maximizes at 510 ug/hr after 1 hour of the test, and then decreases exponentially reaching a minimum of 14 ug/hr of drug. The release rate of drug levels after 5 days ranges from 6 to 10 ug/hr. The ratio of released amount of tylosin tartrate is about 13 w/o.

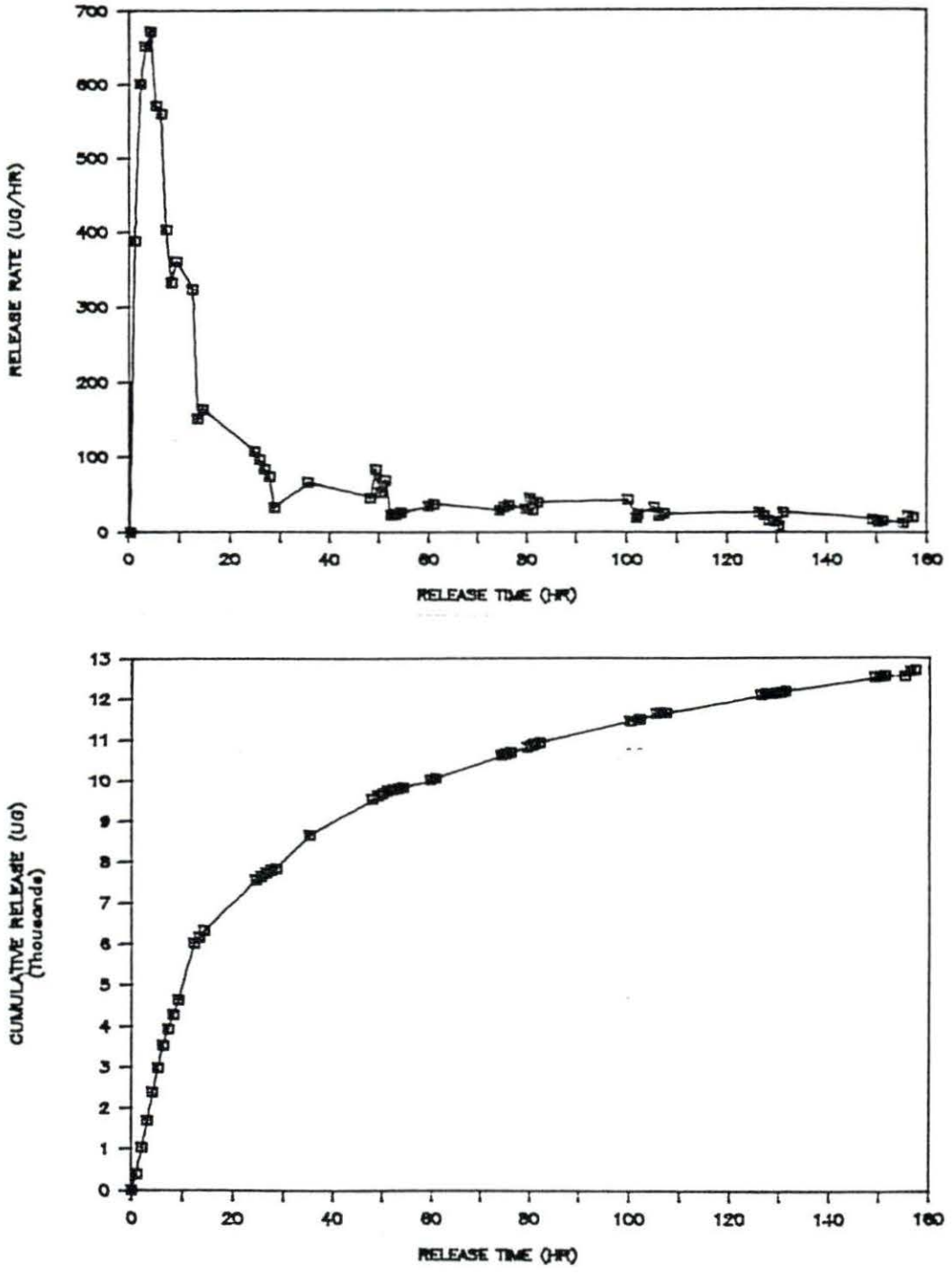

FIGURE 10. Release characteristics from insert D5

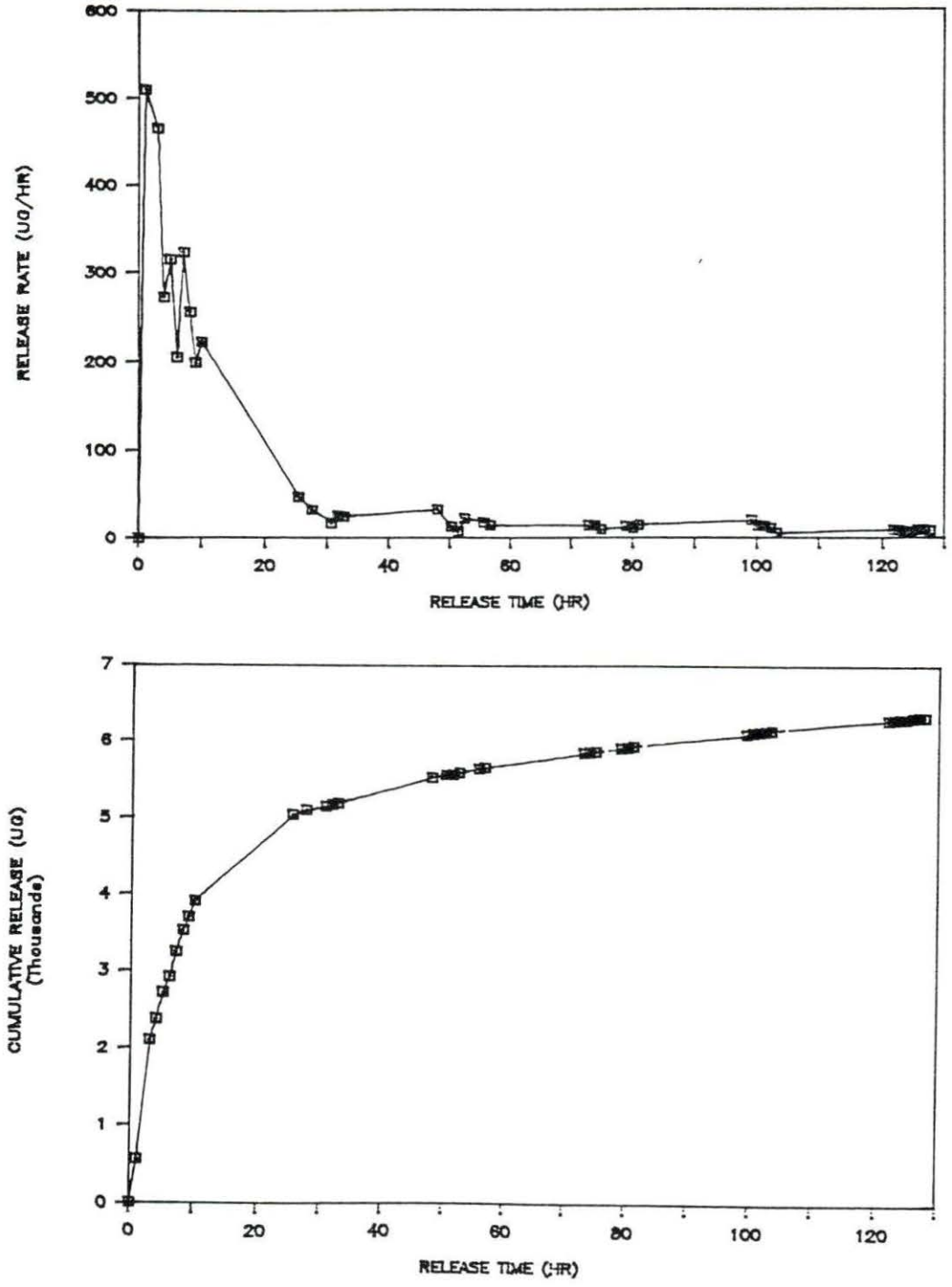

FIGURE 11. Release characteristics from insert D3

Physical Characteristics of the Insert

Before and after the release experiment, each insert was physically checked for structural irregularities using a Nikon stereomicroscope. The ocular insert was cylindrical with irregular edges. The dry sodium alginate matrix was brittle and transparent; the copolymer/drug particles could be seen entrapped and dispersed between the alginate films. The surface of the insert was rough because these particles formed bumps in the dry alginate matrix. Most of the particles were in the contour part of the cylinder.

After crosslinking the sodium alginate insert in calcium chloride solution, the calcium alginate matrix was also transparent and brittle in the dry state. After being immersed in the mammalian Ringer's solution for a few minutes, the insert swelled and formed a jelly-like structure. Bioerosion of the device was noticed; loosened parts of the alginate were floating in the Ringer's solution. Micropores and minor cracks were seen in some parts of the dry alginate matrix.

DISCUSSION

Preparation and Selection of Alginate

After crosslinking the sodium alginate with the desired metal cation, the crosslinked alginates underwent a bioerosion test. Alginates showed weight losses over the 7-day test period. The rate for bioerosion was controlled by the gradual displacement of the crosslinking polyvalent metal ion with a noncrosslinking monovalent ion from the saline (Michaels, 1975). Calcium alginate dissociated completely after five days in the mammalian Ringer's solution; the brittle alginate became tacky and easy to disperse. The calcium alginate was chosen as the matrix material for the bioerodible insert because it would bioerode in the eye in about five days.

Production of the Bioerodible Insert

All the ring devices were fabricated in the same manner. The 10:90 m/o HEMA:MMA copolymer powder dissolved easily in dimethyl formamide. Tylosin tartrate was then added to the copolymer solution where the drug dissolved completely. Neither copolymer nor drug was seen to precipitate from the solution. The solid copolymer/drug transparent precipitate was mixed with liquid nitrogen and crushed.

The bioerodible insert was easy to produce. Fifty milligrams of tylosin tartarte was loaded in the copolymer particles which were then entrapped in the calcium alginate matrix. The loading amount of drug in the matrix can affect the release from a monolithic device (Schacht, 1984). When the sodium alginate ring device was crosslinked in the calcium chloride solution for 12 hours, some of the drug was lost in that solution. Increasing the time of crosslinking could decrease the bioerosion rate of the calcium alginate.

Evaluation of Tylosin Tartrate Release

During the release experiments, all inserts showed similar release characteristics. When the insert was placed in mammalian Ringer's solution, water permeated readily through the matrix. The flow rate of the Ringer's solution was adjusted to 2 ml/hr. The drug within the copolymer particles dissolved in the permeating water forming a solution whose concentration reaches saturation near the center of the copolymer particle but decays gradually as the surface of the particle was approached (Sadek, 1984).

The tylosin tartrate release pattern from the 10:90 m/o HEMA:MMA copolymer particles was similar to that from the copolymer solution tested by Ryoo (1986). This copolymer contained 50 mg of tylosin tartrate. About 60 w/o of the

cumulative drug was released after 24 hours, and around 84 w/o after 2 days. The release was high initially, then decreased exponentially reaching a constant level of release for the remainder of the experiment. After 5 days, the drug release rate ranged from 15 to 29 ug/hr which was above the 1.2 ug/hr required for the treatment of bovine ocular infections. The maximum release rate (1154 ug/hr) was noticed after the first 8 hours.

Each ring device contained 50 mg of tylosin tartrate in the copolymer particles which were dispersed in the calcium alginate matrix. The release characteristics from the ring were also similar to those reported by Ryoo (1986). The maximum release rates (510-672 ug/hr) occurred 1-4 hours after starting the release experiment. After 5 days, the drug release rates were still greater than 1.2 ug/hr. When comparing the release characteristics from the copolymer with those from the insert, a lower peak for the release from the insert was noticed. These peaks occurred at different release times. This difference could be due to the calcium alginate matrix which entrapped the copolymer/drug particles.

In conclusion, findings of this research suggest that production of a bioerodible ocular ring to treat BIK is feasible. This ring device is capable of releasing tylosin

tartrate for more than 5 days and maintaining a release rate above that needed for treatment. The calcium crosslinked alginate degrades in the mammalian Ringer's solution after 5 days, and the copolymer serves its purpose as a regulating membrane of drug diffusion.

RECOMMENDATIONS FOR FUTURE RESEARCH

Although this bioerodible ocular insert gives a release rate of tylosin tartrate above the minimum rate required for treating bovine ocular infections, more studies are needed regarding the use of this device. The final cylindrical configuration of the insert can be modified and improved so as to eliminate any edges or surface roughness in the dry state, (before use, a ring would be preconditioned in saline solution for a few minutes so that the ring would be softened prior to insertion). More bioerosion tests will help in the evaluation of the degradation rate of the prototype insert. In vivo testing is needed to check retention of the ring device in the eye, to evaluate any irritation of the eye, to see if swelling of the insert influences ring retention, and to confirm that in vivo release characteristics will be similar to those seen in the in vitro experiments.

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APPENDIX A: EROSION RATES OF CROSSLINKED ALGINATES

Metal Ion	Testing Day 0	1	2	3	4	5	6	7	8
	Weight of Sample (gm)x10^4								
Na^+	72	0							
	76	0							
	77	0							
Cd^{++}	81	47	0						
	66	46	0						
	80	35	0						
Zn^{++}	68	57	0						
	75	61	0						
	77	55	0						
Ca^{++}	67	51	46	29	18	0			
	70	57	58	49	26	0			
	109	88	88	81	60	0			
	81	63	--	36	--	--	--	0*	
	68	55	--	50	--	--	--	24	0
	73	52	--	50	--	--	--	34	0
Ba^{++}	80	57	54	46	44	44	44	43	
	79	57	--						
	77	55	53	45	45	45	45	45	
Fe^{++}	77	63	63	62	62	61	62	62	
	78	67	56	53	53	55	57	57	
	66	55	68	67	66	68	54	54	
Al^{+++}	83	60	58	53	57	55	56	58	
	80	60	59	53	56	55	57	60	
	81	59	58	53	56	55	57	59	

* all samples are crosslinked for 12 hours, except the last 3 samples of the calcium alginate are each crosslinked for 24, 36, or 41 hours.

APPENDIX B: TYLOSIN TARTRATE RELEASE-EXPERIMENT H4

Release Time (hr)	Volume of Dilution (uL)	Tylosin in Spot (ug)	Total Drug (ug)	Release Rate (ug/hr)	Cumulative Release (ug)
1	100	0	0	0	0
2	1400	0.74	412	412	412
3	2000	1.04	828	828	1240
4	2000	1.18	944	944	2184
5	2000	1.34	1069	1069	3254
6	2000	1.26	1011	1011	4265
8	1900	1.56	1189	1155	6691
9	1900	1.23	932	951	7623
10	1900	1.15	877	877	8500
11	1800	0.99	711	711	9212
24	1500	0.52	314	314	10526
25	500	1.10	220	220	10746
26	500	1.59	309	309	11064
27	500	1.43	285	285	11349
33	500	0.93	185	185	12079
34	300	0.81	97	97	12176
48	100	0.98	39	39	12761
50	100	1.35	54	54	12815
51	100	1.02	41	38	12856
52	100	1.05	42	42	12898
53	100	1.23	49	47	12947
55	300	0.70	84	84	13106
56	100	1.03	41	38	13147
57	100	1.11	44	44	13191
72	100	0.60	24	24	14112
73	100	0.30	12	12	14124
74	100	0.40	16	16	14140
75	100	0.40	16	16	14156
79	100	0.69	27	27	14231
80	100	0.76	30	30	14262
81	100	0.66	26	26	14288
96	100	0.55	22	22	14516
97	100	0.58	23	23	14540
98	100	0.51	20	20	14560
99	100	0.42	17	17	14577
100	100	0.47	18	18	14596
104	100	0.42	17	17	14682
105	100	0.62	24	24	14706
122	100	0.44	18	18	15063
123	100	0.54	22	22	15084
124	100	0.80	32	32	15116
125	100	0.72	29	29	15146

Release Time (hr)	Volume of Dilution (uL)	Tylosin in Spot (ug)	Total Drug (ug)	Release Rate (ug/hr)	Cumulative Release (ug)
126	100	0.51	20	20	15166

APPENDIX C: TYLOSIN TARTRATE RELEASE-EXPERIMENT D3

Release Time (hr)	Volume of Dilution (uL)	Tylosin in Spot (ug)	Total Drug (ug)	Release Rate (ug/hr)	Cumulative Release (ug)
1	500	2.81	561	510	561
3	700	1.67	467	467	2106
4	500	1.36	273	273	2379
5	300	2.83	340	340	2718
6	400	1.28	205	205	2923
7	400	2.02	322	322	3246
8	300	2.30	276	255	3522
9	300	1.50	180	198	3702
10	300	1.76	211	222	3913
25	200	0.58	46	46	5041
28	100	0.80	32	32	5109
31	100	0.43	17	17	5153
32	100	0.64	26	26	5178
33	100	0.64	25	25	5204
48	100	0.82	33	33	5542
50	100	0.35	14	14	5567
51	100	0.20	8	8	5575
52	100	0.61	24	22	5599
56	100	0.47	18	18	5647
57	100	0.35	14	14	5661
73	100	0.36	14	14	5851
74	100	0.37	15	15	5866
75	100	0.25	10	10	5876
79	100	0.36	14	14	5919
80	100	0.29	11	11	5931
81	100	0.39	15	15	5946
99	100	0.52	21	21	6100
100	100	0.35	14	14	6114
101	100	0.35	14	14	6128
102	100	0.29	12	12	6139
103	100	0.14	6	6	6145
122	100	0.25	9	9	6283
123	100	0.19	8	8	6291
124	100	0.20	8	8	6299
125	100	0.16	6	6	6305
126	100	0.25	10	10	6315
127	100	0.23	9	9	6324
128	100	0.22	9	9	6333

APPENDIX D: TYLOSIN TARTRATE RELEASE-EXPERIMENT D5

Release Time (hr)	Volume of Dilution (uL)	Tylosin in Spot (ug)	Total Drug (ug)	Release Rate (ug/hr)	Cumulative Release (ug)
1	900	1.08	389	389	389
2	1100	1.48	650	601	1038
3	1000	1.63	652	652	1691
4	1000	1.70	679	672	2370
5	1000	1.50	600	572	2970
6	1100	1.27	561	561	3531
7	1000	1.01	403	403	3934
8	800	1.11	355	332	4289
9	800	1.12	360	360	4650
12	500	1.63	327	323	6030
13	500	0.75	151	151	6181
14	500	0.82	164	164	6345
25	400	0.67	107	107	7570
26	400	0.60	96	96	7667
27	200	1.05	84	84	7751
28	200	0.92	74	74	7825
29	100	0.84	34	34	7858
35	100	1.66	66	66	7984
48	100	1.15	46	46	9553
49	100	2.10	84	84	9637
50	100	1.34	53	53	9691
51	100	1.73	69	69	9760
52	100	0.60	24	24	9784
53	100	0.68	27	24	9811
54	100	0.66	26	26	9837
60	100	0.88	35	35	10031
61	100	0.96	39	39	10070
74	100	0.76	30	30	10631
75	100	0.87	35	35	10666
76	100	0.91	36	36	10702
79	100	0.33	33	31	10830
80	100	1.14	46	46	10876
81	100	0.76	30	30	10906
82	100	1.02	41	41	10947
100	100	1.11	44	44	11450
101	100	0.50	20	20	11470
102	100	0.62	25	25	11495
105	100	0.84	34	34	11626
106	100	0.56	22	22	11648
107	100	0.65	26	26	11674
126	100	0.68	27	27	12090
127	100	0.58	23	23	12114

Release Time (hr)	Volume of Dilution (uL)	Tylosin in Spot (ug)	Total Drug (ug)	Release Rate (ug/hr)	Cumulative Release (ug)
128	100	0.41	16	16	12130
129	100	0.36	14	14	12145
130	100	0.23	9	9	12154
131	100	0.72	27	27	12183
149	100	0.49	18	18	12526
150	100	0.36	14	14	12541
151	100	0.40	16	16	12556
155	100	0.30	12	12	12549
156	100	0.54	22	22	12670
157	100	0.53	21	21	12692