The preparation of ciprofloxacin hydrochloride-loaded chitosan and pectin microspheres

THEIR EVALUATION IN AN ANIMAL OSTEOMYELITIS MODEL

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Ciprofloxacin hydrochloride-loaded microspheres were prepared by a spray-drying method using pectin and chitosan. The effects of different polymers and drug ratios were investigated.

The most appropriate carriers were selected by *in vitro* testing. A rat methicillin-resistant *Staphylococcus aureus* osteomyelitis model was used to evaluate the effects of the loaded microspheres.

The drug was released rapidly from the pectin carrier but this was more sustained in the chitosan formulation.

Chitosan microspheres loaded with ciprofloxacin hydrochloride were more effective for the treatment of osteomyelitis than equivalent intramuscular antibiotics.

Osteomyelitis is a challenging clinical problem. Systemic treatment requires high serum concentrations of antibiotics for extended periods, with a greater incidence of side-effects, an increased cost and low patient compliance. Local treatment would therefore seem to be ideal with better compliance and fewer side-effects.[1] Antibiotics may be given locally in various ways such as by irrigation systems,[2] venous or arterial perfusion,[3] implantable antibiotic pumps,[4,5] depot delivery systems using polymethylmethacrylate beads,[6] Plaster of Paris,[7] hydroxyapatite cement,[8] polycaprolactone microspheres,[9,10] poly DL-lactide microspheres,[11,12] poly (DL-lactide-co-glycolide) microspheres,[13,14] fibrin clots[15] and collagen-based carriers.[16-19] A biodegradable drug delivery system would have the obvious advantage of eliminating the need for additional surgery to remove the carrier.

Ciprofloxacin hydrochloride is a broad-spectrum fluoroquinolone and is one of the drugs of choice for the treatment of osteomyelitis.[1] It is currently available in tablet form or in parenteral and ophthalmic formulations. It has a plasma half-life of three to five hours and penetrates many body fluids and tissues in therapeutic concentrations.[20] There are few side-effects after long-term treatment and a high concentration at the site of infection. The high bioavailability makes it a preferred agent against susceptible methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Ocular,[21] nasal,[22] topical,[23] microsphere,[24] microparticle,[25] nanoparticle,[26,27] liposome[28] and implant[29,30] formulations of this antibiotic have been investigated.

Pectin and chitosan are natural, hydrophilic, biocompatible and biodegradable polymers with low toxicity. They have been used to prepare microspheres.[31-33] Pectin is an anionic polysaccharide, primarily composed of poly-D-galacturonic acid in which some of the residues are methylesterified.[31,32] Chitosan is a cationic polysaccharide, derived by the deacetylation of chitin.[33]

The microspheres are prepared by spray-drying which is easy and reproducible. This technique provides efficient encapsulation, a narrow size distribution and small particle sizes with low levels of toxic residual organic solvent.[34,35]

We prepared ciprofloxacin hydrochloride-loaded microspheres using chitosan and pectin. The surface morphology of the microspheres, the influence of the drug-polymer ratio on the formation of the microspheres, the drug-loading capacity and the particle size were investigated. The formulations were characterised using an *in vitro* release study. Those providing sustained drug release were selected for an *in vivo* study.

Our aim was to prepare microspheres using biodegradable polymers for the sustained release of ciprofloxacin hydrochloride and to prove its efficacy in a rat model of implant-related osteomyelitis.

Materials and Methods

Preparation of microspheres. We prepared ciprofloxacin hydrochloride (Kocak Farma Com-

pany, Istanbul, Turkey) loaded chitosan (Sigma-Aldrich, Munich, Germany) microspheres (CMS) with a polymer:drug ratio of 1:1 (w/w) and ciprofloxacin hydrochloride-loaded pectin (Sigma) microspheres (PMS 1-4) with polymer:drug ratios (w/w) of 1:1, 2:1, 3:1, 4:1. Chitosan was dissolved in 1% (v/v) acetic acid (Merck KGaK, Darmstadt, Germany) solution to obtain a polymer solution at a concentration of 0.5% (w/v). We dissolved 2% (w/v) of pectin in distilled water. The prepared solutions were sprayed through the nozzle of a mini spray dryer (Model 191, Büchi Labortechnik AG, Flawil, Switzerland). The microspheres were collected and weighed to determine the production yield. Each formulation was carried out in triplicate. Blank microspheres were prepared for comparison as controls.

Characterisation of the microspheres. Thermal analyses were carried out on ciprofloxacin hydrochloride, pectin, chitosan, drug-loaded microspheres and blank microspheres, using a differential scanning calorimeter (DSC; Model Q100, TA Instruments, New Castle, Delaware). The encapsulation efficiency and actual drug content of the microspheres were calculated. Analysis of particle size was performed using a Malvern Mastersizer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The d_{50} (mean diameter of microspheres) values for all formulations were expressed as the mean particle size range of microspheres.

The surface morphology of the microspheres was studied using Scanning Electron Microscopy (JXA 840A; JEOL-USA Inc., Peabody, Massachusetts).

***In vitro* drug release**. *In vitro* release profiles of ciprofloxacin hydrochloride from CMS and PMS1-4 microspheres were examined in phosphate buffer (pH: 6.40). We placed 1.0 ml of dissolution medium into ependorph tubes to which 10 mg of ciprofloxacin hydrochloride-loaded microspheres were added. The tubes were placed in a Forma orbital shaker (Thermo Electron Corporation, Milford, Massachusetts) at 37°C at 120 rpm. At scheduled time intervals, the tubes were taken and centrifuged at 5000 rpm for five minutes; 30 µl samples were withdrawn and replaced with fresh medium. The samples were diluted with the same buffer and analysed spectrophotometrically at 271 nm. The *in vitro* release studies for all formulations were carried out in triplicate.

***In vivo* studies**. Male Wistar albino rats were used for the animal model. All investigations were performed according to the EU guidelines for animal experimentation.[36] The protocol was approved by the Animal Care Committee of our institution.

The selected formulations (CMS, PMS-4) and blank microspheres were placed in vials, sealed and γ-irradiated (25 kGy) using ^{60}Co as the radiation source. The sterility of the microspheres was checked according to United States pharmacopoeia sterility test XXIV procedure. The sterile microspheres were kept in sealed vials until required.

Implant-related osteomyelitis was established using a modified Norden foreign-body model of experimental osteomyelitis with a Kirschner wire.[37,38] Sodium morrhuate was not used as a sclerosant agent. The rats were randomly divided into four groups of eight.

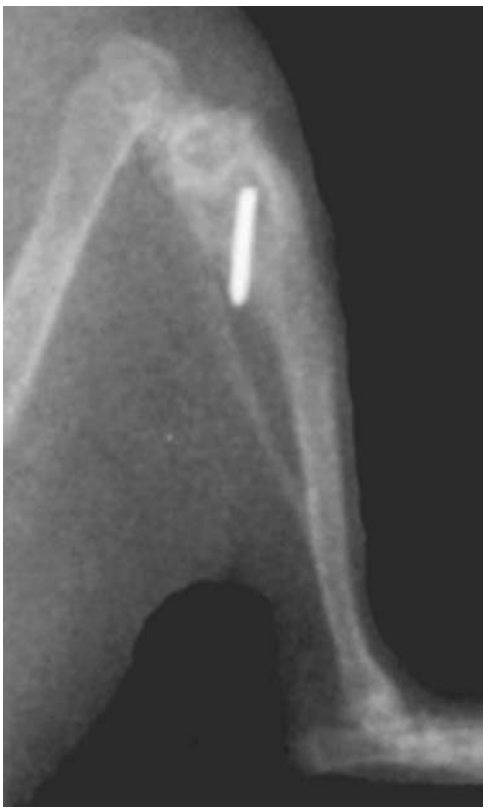

Fig. 1

Post-operative lateral radiograph at week 6 showing bone lysis and a Kirschner wire in the proximal defect in the tibia.

The proximal tibial metaphysis was exposed through an anteromedial incision. A cavity was opened using a dental burr and a Kirschner wire (5.0 x 1.0 mm) was inserted into this. A suspension (0.2 ml) containing 1.0 x 10^7 colony-forming units/ml of an osteomyelitis strain of MRSA, sensitive to ciprofloxacin, was injected into the defect. The cavity was sealed by applying a small amount of dental gypsum. The fascia was repaired with a continuous chromic catgut suture and the subcutaneous tissue and skin were closed by interrupted sutures. Lateral radiographs (Fig. 1) were taken before and at intervals of two weeks after surgery and for the duration of each experiment. In the fourth week, a scan with MDP ^{99}Tc was used and the ESR (mm/h) and level of C-reactive protein (mg/l) were also evaluated. After six weeks the Kirschner wires were extracted. The mean pH of the area of osteomyelitis was measured. Most of the rats had an encapsulated abscess subcutaneously which was excised. The infected area in the bone was also excised and washed with saline solution.

In the CMS and PMS-4 groups, sterile drug-loaded microspheres which were equivalent to an intravenous dose

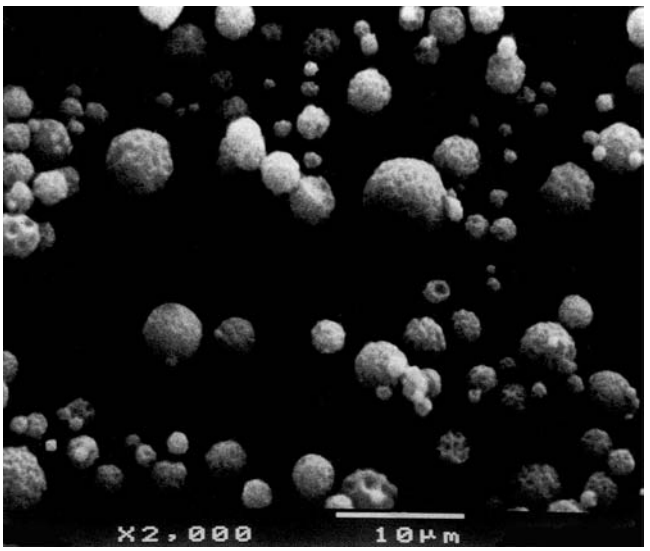

Fig. 2a

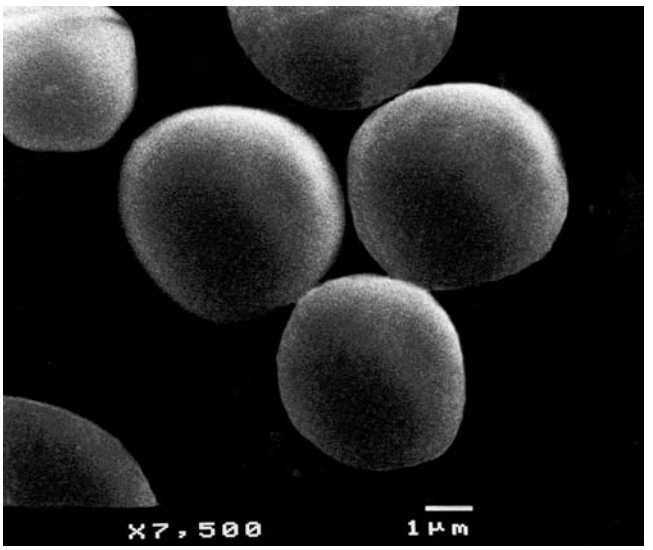

Fig. 2b

Scanning electron microscope of a) ciprofloxacin hydrochloride-loaded chitosan microspheres (x2000) and b) ciprofloxacin hydrochloride-loaded pectin microspheres (x7500).

Table I. The production yield, actual drug content, encapsulation efficiency and particle size of ciprofloxacin hydrochloride-loaded microspheres

Formulation*	Polymer type	Polymer:drug ratio	Production yield (%; SD)	Theoretical drug content (%)	Actual drug content (%; SD)	Encapsulation efficiency (%; SD)	Particle size (µm; SD)
CMS	Chitosan	1:1	*48.21* (2.94)	*50*	*50.47* (0.64)	*100.94* (1.29)	4.71 (0.38)
PMS-1	Pectin	1:1	*46.35* (4.82)	*50*	*48.44* (1.46)	*98.88* (2.93)	3.97 (0.21)
PMS-2	Pectin	2:1	*47.93* (3.09)	*33.33*	*33.21* (0.92)	*99.64* (2.77)	4.06 (0.29)
PMS-3	Pectin	3:1	*46.68* (3.62)	*25*	*25.81* (0.49)	*103.27* (2.01)	4.29 (0.36)
PMS-4	Pectin	4:1	*45.57* (5.43)	*20*	*20.24* (0.40)	*101.21* (2.02)	4.52 (0.25)

* CMS, ciprofloxacin hydrochloride-loaded chitosan microspheres; PMS, ciprofloxacin hydrochloride-loaded pectin microspheres

of ciprofloxacin hydrochloride (62 mg ciprofloxacin hydrochloride/kg weight of the rats) for ten days were inserted into the bony defect. Another group was treated with an intramuscular injection of ciprofloxacin hydrochloride (6.2 mg/kg/day) for 21 days as a comparison. Sterile blank microspheres were also implanted in a control group. In all groups, the subcutaneous tissue and skin were closed by interrupted sutures.

After completion of the antimicrobial therapy, the rats were killed by a lethal dose of ether. The infected bones were removed aseptically, weighed and frozen. These frozen bone samples were ground, suspended in 1 ml of saline, and diluted serially. Aliquots (0.1 ml) were plated on Mueller-Hinton agar plates and incubated at 37°C overnight. The plates were examined for purity and colony morphology. The colonies were counted and the colony-forming units per gram of bone were calculated. The results were expressed as the mean colony-forming units per gram of bone for each treatment group.

Histopathological analysis. Bone samples were removed for histopathological examination, placed in a fixative solution of 10% formalin for one day, decalcified in a 10% nitric acid solution, washed with water and embedded in paraffin. Longitudinal sections 4 to 5 µm thick were obtained using a standard rotary microtome and stained with haematoxylin and eosin. The samples were examined under light microscopy. Acute inflammatory cells (polymorphonuclear cells), chronic inflammatory cells (mononuclear cells), the presence of giant cells, proliferation of fibroblasts and increase in capillaries were evaluated separately. Each item was graded by a single, blinded, pathologist (MA) by a semi-quantitative approach as absent (0), mild (1), moderate (2) and severe (3).

Statistical analysis. *In vitro* release data obtained from each experiment and the relationships between the *in vivo* results were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparisons test. In the histological studies the differences between the groups were tested for significance by the chi-squared test. A p value of < 0.05 was considered to be significant.

Results

For all formulations, the production yield was low (46% to 48%), the particle diameters were less than 5 µm and the encapsulation efficiencies were very high (> 98%) (Table I). In the differential scanning calorimeter curves of micro-

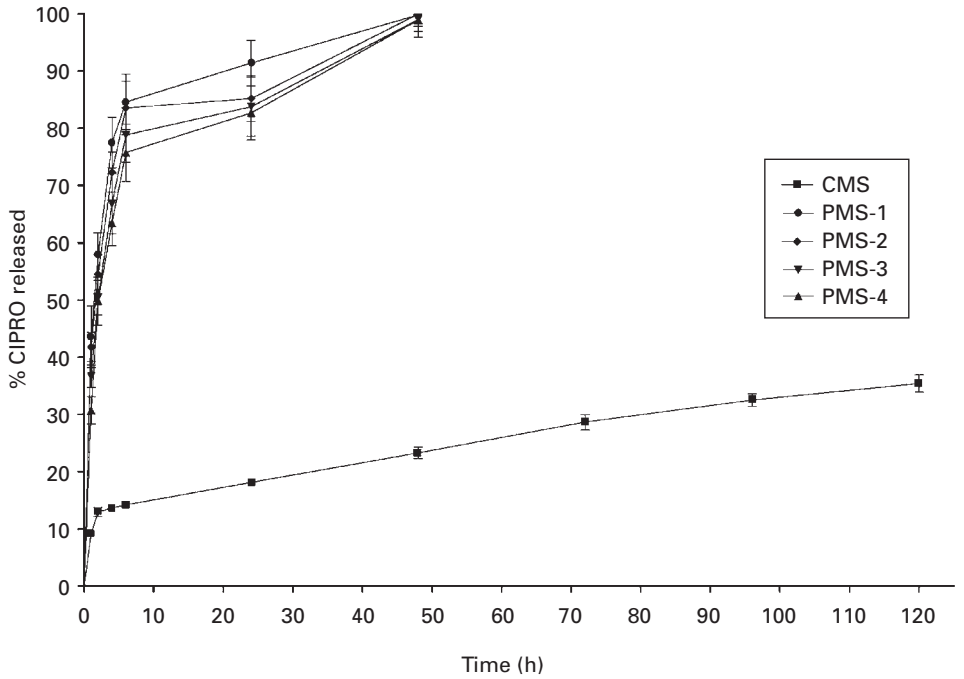

Fig. 3

Graph showing *in vitro* release profile of ciprofloxacin hydrochloride from drug-loaded microsphere formulations.

spheres, characteristic peaks of chitosan and pectin were visible and ciprofloxacin hydrochloride was present as an amorphous compound. There were no interactions between the polymers and ciprofloxacin hydrochloride. According to the scanning electron microscopy measurements, all microsphere formulations were spherical. The CMS microspheres had a porous surface and PMS1-4 microspheres a smooth surface (Fig. 2). The release profiles of ciprofloxacin hydrochloride from CMS and PMS1-4 are shown in Figure 3. There was a significant difference in the rates of dissolution ($p < 0.001$). The pectin microspheres showed an initial burst effect which increased as the pectin:ciprofloxacin hydrochloride ratio decreased. These differences in the pectin microspheres were not significant ($p > 0.05$). The full amount of ciprofloxacin hydrochloride was released from PMS1-4 microspheres in 48 hours but only 35.4% was released from CMS microspheres in 120 hours. Based on these results, CMS and PMS-4 were selected for *in vivo* studies. The mean number of colonies per gram of bone are shown in Figure 4. In two of the eight specimens in the CMS group there was no growth. The difference between the CMS and the other groups was statistically significant ($p < 0.001$), but that between the intramuscular and control groups was not ($p > 0.05$).

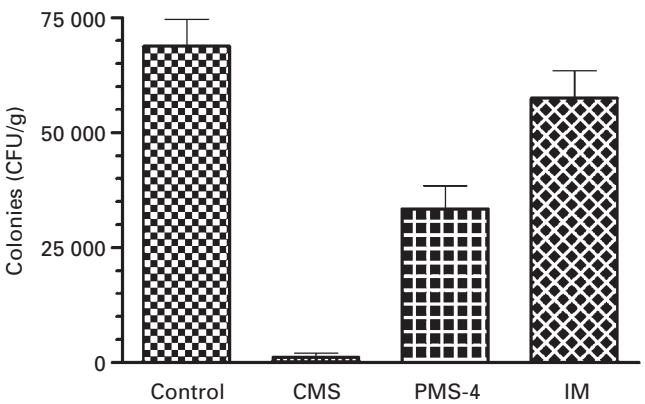

Fig. 4

The mean methicillin-resistant *Staphylococcus aureus* colony forming units (CFU) per gram of bone sample after treatment for 21 days.

The differences between all of the groups were significant in terms of the acute inflammation ($p < 0.05$), chronic inflammation ($p < 0.05$) and increase in capillaries ($p < 0.05$). The CMS group showed less inflammation and fibrosis with an increase in capillaries compared with the PMS-4, intramuscular and control groups (Figs 5 and 6). Significantly less necrotic bone and an increase in multinuclear giant cells were seen in the CMS group compared with the control group, but these results were not statistically significant ($p > 0.05$).

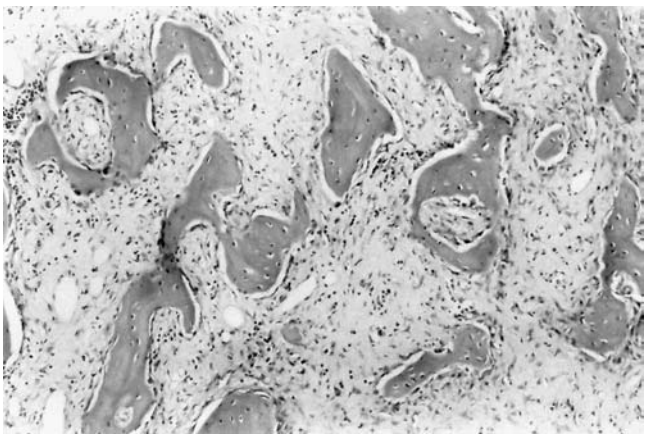

Fig. 5

Photomicrograph of trabecular bone in the CMS group showing less inflammatory infiltrate which involves plasma cells and neutrophils (haematoxylin and eosin, x100).

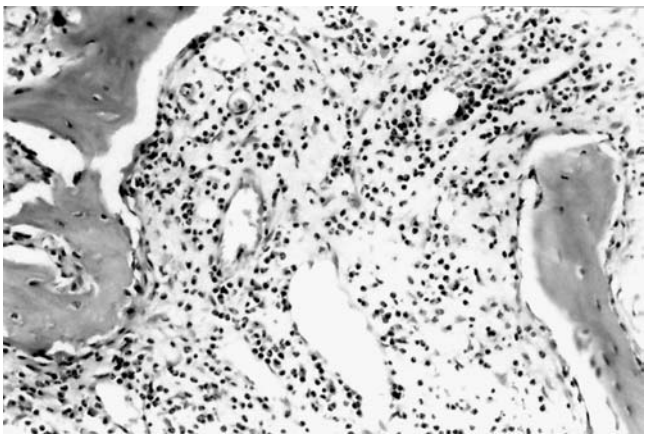

Fig. 6

Photomicrograph of trabecular bone in the control group showing severe mixed-type inflammatory cells which involve plasma cells, lymphocytes and neutrophils (haematoxylin and eosin, x100).

Discussion

Bone infections are difficult to treat.[39] Controlled and prolonged local release of antibiotics may solve the problems associated with systemic treatment and achieve high local concentrations of antibiotic while maintaining low systemic levels.[40]

Many local methods of delivery of antibiotics in non-biodegradable and biodegradable systems have been evolved for the treatment of infected bone. Non-biodegradable systems include polymethylmethacrylate bone cement and beads,[5,7,41] glass ceramic blocks,[42] hydroxyapatite blocks,[7,43] biodegradable systems of collagen-gentamicin sponge,[15] poly(3-hydroxybutyrate-co-3-hydroxyvalerate) rods,[44,45] poly (DL-lactide) microspheres[10,11] and poly (DL-lactide-co-glycolide) implants.[46-49]

Recently, several local drug delivery systems using biodegradable materials have been studied in animal models of implant-related osteomyelitis and have shown good results.[11,50,51]

Fluoroquinolones are the drugs of choice for the treatment of bone infections because of their favourable penetration of poorly vascularised sites, their advantageous bactericidal effects against all probable bone pathogens and lack of serious adverse reactions.[52] They are very active against the biofilms of *Staph. aureus* and *Pseudomonas aeruginosa*.[53,54]

In our study we omitted sodium morrhuate in order to produce a model with very little dead bone. This allows better penetration of antibiotics. However, satisfactory use of this model does not imply that the technique would be effective in situations in which there is dead bone.

Foreign-body-type multinuclear giant-cell formation was seen in the specimens. Residual microsphere particles were detected slightly more often in the PMS-4 group compared with the CMS group. More multinuclear giant cells were seen in the CMS group compared with the control group, but these results were not statistically significant. Histological analysis confirmed that the microspheres were biodegradable and did not impede formation of new bone. We do not know why CMS produce more giant cells but the role of chitosan in the formation of giant cells remains to be determined.

In our study, only two of eight animals in the CMS group were sterilised by the technique. Colonies were grown from all other tibiae. The failure to eradicate the infection from six of eight of the tibiae is important.

Studies of methylmethacrylate carriers have shown that most of the antibiotic is permanently trapped in the cement and cannot be released.[55] Our results showed that the biodegradable microspheres are a more efficient delivery system. The optimal dose of applied antibiotics and the effects of resistance of the bacteria need to be investigated further in these delivery systems.

Supplementary Material

Tables showing *in vivo* results of treatment by ciprofloxacin hydrochloride-loaded microspheres and intramuscular injection and the histological changes in the four groups are available with the electronic version of this paper on our web site at www.jbjs.org.uk

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