Journal of
Pharmacy and Pharmacology

JPP 2003, 55: 559–566

Received August 27, 2002
Accepted January 20, 2003
DOI 10.1211/002235702883
ISSN 0022-3573

In-vitro and in-vivo antibacterial activity evaluation of a polyurethane matrix

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Abstract

Various in-vitro and in-vivo methods for evaluation of the duration of antibacterial activity were compared using a controlled-release polyurethane matrix developed for the prevention of surface bacterial adhesion and growth. Cefadroxil was incorporated into this polyurethane matrix by a solvent casting method before the matrix was coated with polyurethane in tetrahydrofuran solution. The release of cefadroxil from the matrix into distilled water at 37 °C was measured by HPLC. The morphological change of matrices before and after release studies was investigated by scanning electron microscopy (SEM). The duration of antimicrobial activity of the matrix against *Escherichia coli* and *Staphylococcus aureus* was evaluated by measuring the diameters of the inhibition zone and the optical density of the broth. The matrices were also implanted subcutaneously in rats and the duration of the antibacterial activity was determined by measuring the inhibition zone. The results showed that duration of antibacterial activity of the polyurethane matrix was successfully determined in-vitro by these methods, and the results differed from the conventional in-vitro release study. It was also possible to determine the duration of action of the matrix in-vivo by implanting the matrix in rats, and then measuring the antibacterial activity of the matrix at predetermined time intervals. While a good correlation was observed between the in-vitro and in-vivo methods used in this study to evaluate the duration of the antibacterial activity of the polymeric matrix, the conventional in-vitro release study did not coincide with these results.

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Funding: This work was supported by Korea Research Foundation Grant (KRF-99-041-F00321).

Introduction

Bacteria in nature have a remarkable ability to adhere to the surfaces of biomaterials and form biofilms. The phenomenon wherein bacteria attach to medical implants and cause infection is referred to as device-associated or biofilm-related infection (Elek & Conen 1957). Once formed, it is extremely difficult to eradicate bacteria, even with vigorous antibiotic treatments (Anwar et al 1992; Segreti & Levin 1989). Biomaterial-centred infections in orthopaedic and general surgery, as well as in catheterizations, are a significant problem, frequently resulting in severe morbidity, amputation or even death (Dougherty & Simmons 1982; Gristina 1987). These infections tend to persist until the implant (or foreign body) is removed. Thus, infection caused by implanted polymeric devices is of increasing medical importance.

Most often, eradication of the microorganisms is possible when the infected devices are removed, followed by long-lasting chemotherapy and implantation of a new system (Bayston et al 1989). Preoperative, postoperative and preventive topical application of antibiotics to the wound and polymer can only minimize, and not prevent, bacterial colonization. Thus, alternative strategies for the prevention of foreign-body infections are required. Inhibition of pathogenesis and subsequent protection mechanisms is possible by killing bacteria during the very first steps of colonization. The continuous delivery of sufficient doses of antibiotics on the surface of biomaterial was proposed as a very promising and advantageous approach (Bayston et al 1989).

One of the methods most frequently utilized in an attempt to reduce the incidence of infections was to disperse anti-infectious substances in polymeric matrices where the polymer is to act as a rate-controlling barrier (Schierholz et al 1997). Use of such

preparations may result in a number of benefits, such as reduction of the frequency of dosing, lowered adverse reactions and improved patient compliance (Uchida & Goto 1988).

Conventionally, in-vitro release study has been most commonly used to evaluate the efficacy of these devices. This method determines the amount and the rate of antibiotics released into the media from the biomaterials. However, the duration of antibacterial activity of the devices themselves may not be related with the in-vitro release of antibiotics, since the antibiotics on the surface of the biomaterial are not detected but are enough to inhibit the infection of the devices. Previously, we have reported the effect of so-called pore formers on the controlled release of an antibacterial agent from a polymeric device, and determined the duration of antibacterial activity of the device itself by continuously measuring the diameter of the inhibition zone while transferring the matrix to fresh culture plates after 24 h of incubation (Kim et al 2000). In this study, we compared various in-vitro and in-vivo assay methods reported in the literature to determine the duration of antibacterial activity of a polyurethane matrix. Cefadroxil was chosen as a model drug for its broad antibacterial spectrum against both Gram-positive and -negative bacteria (Uchida et al 1992). *Staphylococcus aureus* and *Escherichia coli* were selected to determine the antibacterial activity of the device since they are the typical Gram-positive and -negative bacteria, respectively, and are also known to be a significant cause of the infections associated with biomaterials (Hogt et al 1986; Solomon & Sherertz 1987). Also, the matrices were coated to sustain the release of cefadroxil from the polyurethane matrices.

Materials and Methods

Materials

Cefadroxil was provided by Cho-A Pharmaceutical Co. (Pusan, Korea). Polyurethane resin (Lot no. 6193) was received from Dong-Sung Chemical Co. (Suwon, Korea). Bovine serum albumin (BSA) and tetrahydrofuran (THF) were purchased from Sigma Chemical Co. (St Louis, MO). All other reagents were special reagent grade and used as purchased. *Escherichia coli* (NIHJ) and *Staphylococcus aureus* (ATCC 25923) were obtained from the microbiology laboratory of Pusan National University (Pusan, South Korea). Mueller-Hinton II agar, trypticase soy broth and blood agar plate (BAP) were purchased from Becton Dickinson and Company (Cockeysville, MD). Male Sprague-Dawley (210~230 g) rats were obtained from Dae-Han Laboratory Animal Research Center Co. (Dae-Jeon, Korea).

Preparation of polyurethane matrices

Matrices were fabricated by the solvent casting method, as previously reported (Kim et al 2000). Briefly, 2 g of cefadroxil and 2 g of pore former (BSA) (1:1 weight ratio) were completely dissolved in 170 mL of double-distilled water. The mixed aqueous solution was lyophilized and ground with a mortar and pestle. The powder was fractionated using the microsieve (Fisher Scientific, Pittsburgh, USA) and the fraction of 62–88 μm particle size was collected. Polyurethane resin (0.6 g) was dissolved in 10 g of THF with constant shaking for 8 h at 70 °C, and then mixed with 0.4 g of lyophilized powder to make 40% (w/w) powder in polymer matrix (equivalent to 20% pore former). This mixture was degassed with an aspirator and poured into a Teflon mould. The solvent was evaporated overnight at ambient temperature followed by vacuum drying for another 48 h. Final matrices were trimmed to 1.0 cm^2 and stored at 4 °C until used.

Coating the matrices

The matrices were coated by dipping them into polymer solution, as reported in the literature (Göpferich 1997). The coating solution was the polyurethane dissolved in THF (1:40, w/w). The dipping was repeated 3 or 10 times. Between the individual coating steps, the matrices were dried on Teflon plate. After the last dipping, the matrices were vacuum-dried at ambient temperature for 7 days.

In-vitro cefadroxil release studies

The matrices were placed in vials containing 20 mL of double-distilled water and placed in a shaking water bath at 37 °C. Samples of medium were taken at predetermined time intervals and replaced with the same amount of fresh water. High-performance liquid chromatography (HPLC) was used for the quantitative determination of cefadroxil in the samples. The amount of cefadroxil released was expressed as the percent of the starting amount in the matrix. Triplicate experiments were conducted for each formulation using three samples taken from one polyurethane matrix.

HPLC analysis of cefadroxil

Cefadroxil concentration was determined using HPLC with a binary pump system (Gilson Model 305 and 306) and automatic injector (Gilson Model 234). A Merck C_{18} LiChroCART 125-4 column (5 μm, 125 × 4 mm; Merck, Darmstadt, Germany) was used as analytical column at ambient temperature. The mobile phase was 10% (v/v) methanol in 10 mM acetate buffer (pH 4.8) at a flow rate of 1.0 mL min^{-1}. The variable wavelength ultraviolet detector (Gilson Model 118) was set at 240 nm. Injections of 20 μL were made for all solutions to be analysed. The retention time of cefadroxil was about 7.9 min.

The calibration curve of cefadroxil was linear ($r^2 = 0.999$, $n = 4$) over the 1.0–200 $\mu g\,mL^{-1}$ range, and the detection limit was determined to be 1.0 $\mu g\,mL^{-1}$. The intra-day and inter-day precision were assessed by analysing four replicates each day for four days, and the coefficient of variation ranged from 3.2 to 8.5% and 8.1 to 17.5%, respectively, at different concentration ranges.

Morphological analysis of matrices

Scanning electron microscopy (SEM) was performed on Au sputter-coated samples before and after release studies using the Hitachi S-4200 SEM (Tokyo, Japan). The surface and cross-section of the matrices were carefully examined to study the effect of coating on the formation of pores/channels in the matrices.

Biological in-vitro release studies

The duration of antibacterial activity of the matrices against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) was determined using both agar and broth. In studies using the agar, a standard streaking method (Acar & Goldstein 1986) was used with slight modification. Briefly, 30 mL of Mueller-Hinton II agar was placed into a culture plate (87 mm diameter, 15 mm height) and solidified. A sterile cotton swab was dipped into the bacterial broth suspension with an optical density of 0.400 at UV 660 nm, and excess fluid was removed by rotating the swab several times against the wall of the vessel. The inoculum was streaked evenly in 3 planes onto the surface of the agar. Then, one polymeric matrix was placed at the centre of each plate and gently pressed down to ensure contact. The diameters of the inhibition zone were measured after incubating the plates for 24 h at 37 °C. The duration of the antibacterial activity of each matrix was investigated by repeatedly transferring the matrices to a freshly prepared plate after 24 h of incubation at 37 °C until the inhibition of bacterial growth was no longer noticed, as reported in the literature (Kim et al 1989). When a plain matrix without cefadroxil was used as a control, no inhibition of bacterial growth was observed.

In studies using broth, a polymeric matrix was immersed into the broth (3.0 mL) in a culture tube, and placed in a 37 °C shaker (Taitec BR-15, Japan) at 150 rev min^{-1} for 24 h. Then, the broth was inoculated with 30 μL of bacterial suspension (*S. aureus* or *E. coli*) with an optical density of 0.400 at UV 660 nm and incubated for 12 h. The antibacterial activity of cefadroxil released from the matrices was determined by measuring the optical density of the broth using UV spectroscopy at 660 nm, while that of clear broth was used as a blank. To determine the duration of the antibacterial activity of the matrices, the matrix was transferred into a fresh broth in a culture tube after 24 h of shaking in the broth. These procedures were continuously repeated until the inhibition of bacterial growth was no longer noticed. In a preliminary study, when *S. aureus* or *E. coli* was inoculated in the broth without cefadroxil, the optical density of the broth at 660 nm after 12 h of incubation at 37 °C was 0.8 and 1.0, respectively.

Each study was performed in triplicate (i.e., three samples taken from one polyurethane matrix).

In-vivo antibacterial activity studies

The duration of antibacterial activity of the matrices was determined in-vivo by modifying the method in the literature, which investigated the half-life of an antibiotic-releasing polymer in mice (Solomon & Sherertz 1987). Briefly, Sprague-Dawley male rats (210~230 g) were anaesthetized using ether. The back of each rat was shaved with clippers, after which a small incision was made in the hairless skin area and a matrix was inserted into the subcutaneous space. The incision was then closed using a single silk suture. The wound area was swabbed with a povidone-iodine solution. Up to 10 matrices were randomly inserted in the back of a rat, then each matrix was removed at predetermined time intervals for up to 24 days. The matrices removed from the back were placed on agar plates streaked with *S. aureus* or *E. coli*, after which the inhibition zone sizes were measured after 24 h incubation at 37 °C.

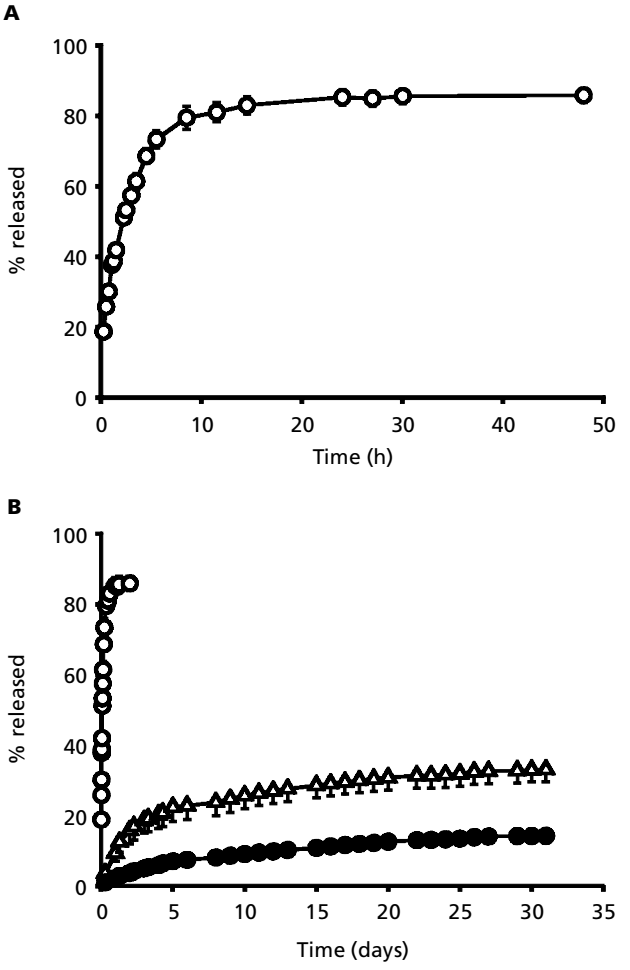

Figure 1 A. In-vitro release profile of cefadroxil from a polyurethane matrix without coating. B. The effect of coating on in-vitro release of cefadroxil from the polyurethane matrices. The matrix contains 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil 1:1), equivalent to 20%(w/w) pore former, without coating (○), with 3 coatings (△) or 10 coatings (●) by dipping in polyurethane–THF (1:40 w/w) solution. Each point represents the mean ± s.d. of three experiments.

Statistical analysis

All experiments were performed in triplicate except for the in-vivo antibacterial study, which was repeated 4 times. The observed data were expressed as mean ± standard deviation (s.d.). For the in-vitro release study, linear regression of the log fractional release against the log time was conducted to determine the release exponent (n) of the following power law expression:

$$M_t/M_\infty = kt^n \quad (1)$$

where M_t is the amount of cefadroxil released at time t, M_∞ is the total cefadroxil released over a long time period, k is the kinetics constant and n is the mechanism of cefadroxil release. Correlation coefficient r^2 was determined to express linearity.

Results and Discussion

In-vitro release of cefadroxil

Figure 1 shows conventional in-vitro release profiles of cefadroxil from polyurethane matrices containing 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil 1:1), equivalent to 20% (w/w) BSA as a pore former. The release of cefadroxil from the matrix without coating was fast, and almost 90% of cefadroxil was released within the first 24 h (Figure 1A). The value of n for $M_t/M_\infty < 60\%$ was 0.46 ($r^2 = 0.998$) without coating, which suggests that the release of cefadroxil from the matrix can be referred to as a Fickian release. However, when the matrix was coated by dipping in polyurethane–THF (1:40 w/w) solution 3 or 10 times, the release rate of cefadroxil dramatically decreased (Figure 1B). After 10

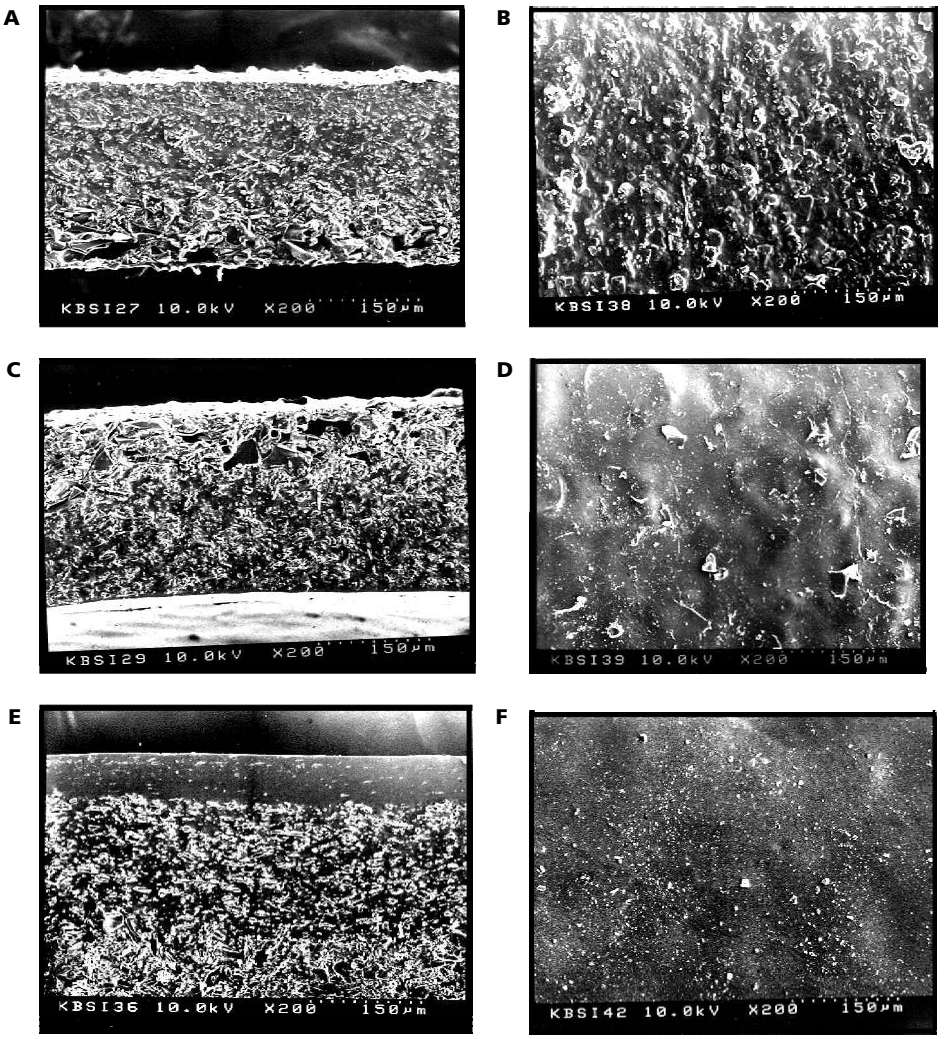

Figure 2 SEM (×200) of cross-section (A, C, E) and surface (B, D, F) of polyurethane matrices without coating (A, B) and after 3 coatings (C, D) or 10 coatings (E, F) produced by dipping in polyurethane–THF (1:40 w/w) solution. Each matrix contains 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil, 1:1), equivalent to 20%(w/w) pore former.

coatings, the n value determined from the linear regression of 5–31 days of release profile was 0.98 ($r^2 = 0.989$), which implies that the release of cefadroxil almost followed a zero-order release kinetics.

Morphological analysis of matrices

SEM of the cross-section of the matrices showed the formation of the coating layer on the surfaces (Figure 2A, C, E), which became smoother as the number of coatings increased up to 10 (Figure 2B, D, F). These results are well correlated with the reduction of release rate in in-vitro release studies (Figure 1), and suggest that the coating layer worked as a barrier for the release of cefadroxil from the polyurethane matrix.

At higher magnification (×1000), typical channel formation was observed in the cross-section of the matrix without coating, after 48 h of release study (Figure 3A, B). However, the channel formation was not significant even after 48 h of release study when the matrix was coated (Figure 3D, F). These results are consistent with the in-vitro release study (Figure 1), which showed almost complete release within 48 h when the matrix was not coated and incomplete release after 3 or 10 coatings.

Biological in-vitro release studies

Agar method

The antibacterial activity of polyurethane matrices, determined using agar, against *S. aureus* and *E. coli* is shown in Figure 4. All matrices produced a zone of inhibition when placed in plates overlaid with *S. aureus* or *E. coli*, while the control polymer matrix without cefadroxil showed no inhibition zone (data not shown). Also, neither the concentration of bacterial suspension nor the size of the matrix affected the diameter of the inhibition zone after 24 h incubation.

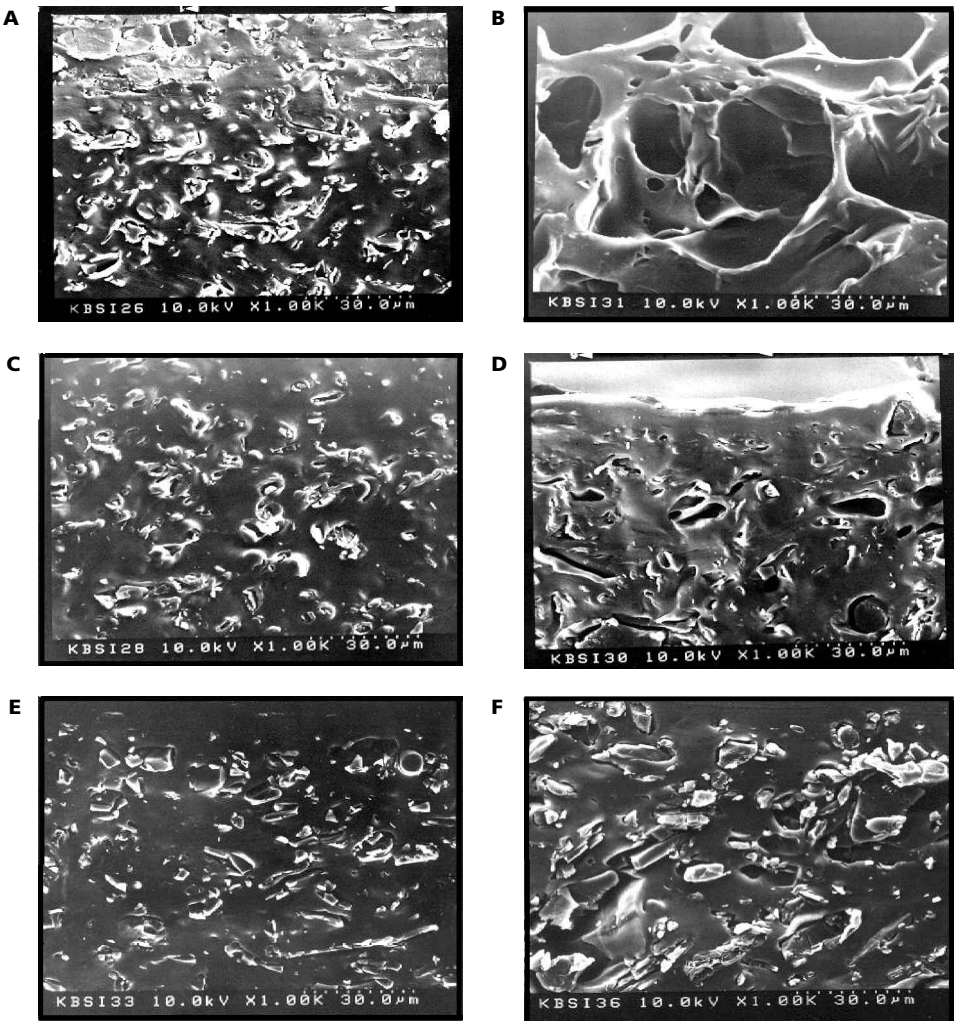

Figure 3 SEM (×1000) of cross-section of polyurethane matrices before (A, C, E) and after (B, D, F) in-vitro release study for 48 h. Each matrix contains 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil, 1:1) without coating (A, B) or with 3 coatings (C, D) or 10 coatings (E, F) produced by dipping in polyurethane–THF (1:40 w/w) solution.

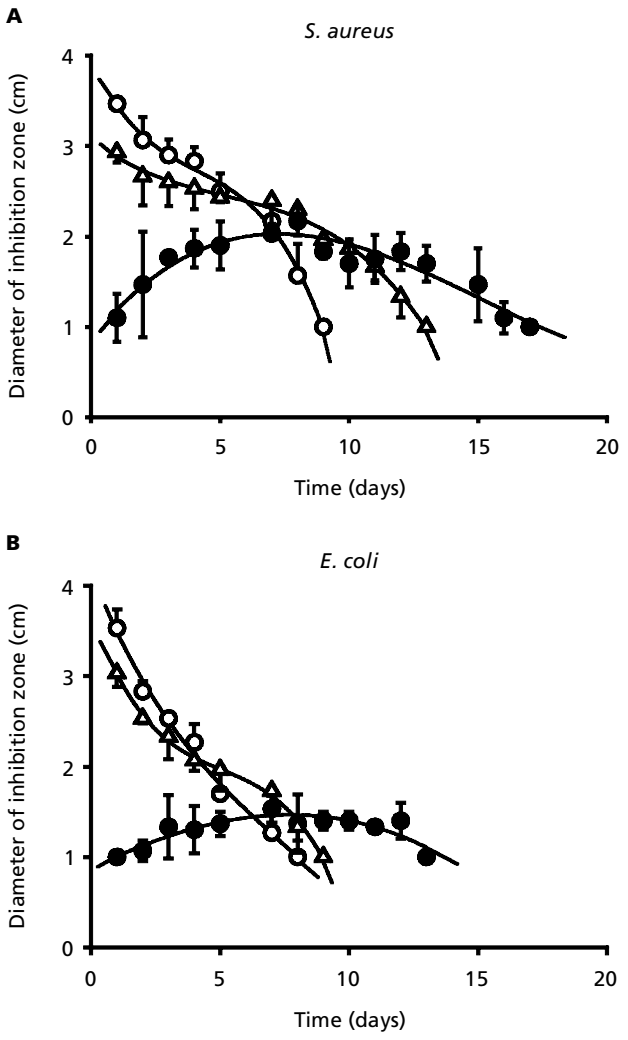

Figure 4 Duration of antibacterial activity of polyurethane matrices against *S. aureus* (A) and *E. coli* (B) determined by using agar. The matrix contains 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil, 1:1) without coating (○) or with 3 coatings (△) or 10 coatings (●) produced by dipping in polyurethane–THF (1:40,w/w) solution. Each point represents the mean ± s.d. of three experiments.

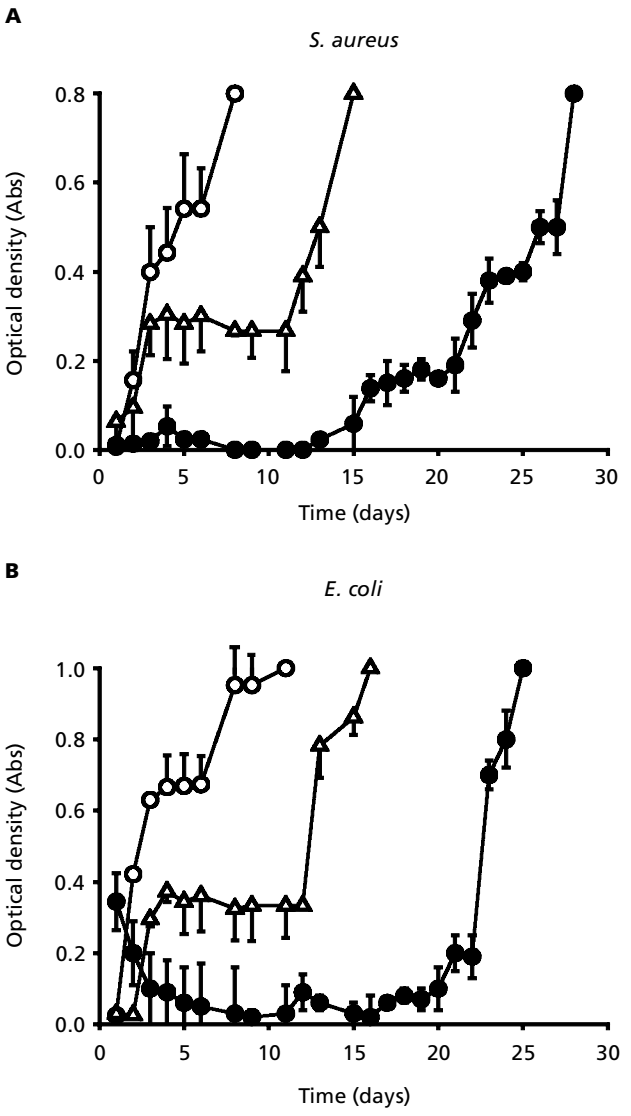

Figure 5 Duration of antibacterial activity of polyurethane matrices against *S. aureus* (A) and *E. coli* (B) determined by using broth. The matrix contains 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil, 1:1) without coating (○) or with 3 coatings (△) or 10 coatings (●) produced by dipping in polyurethane–THF (1:40 w/w) solution. When *S. aureus* or *E. coli* was inoculated in the broth without cefadroxil and incubated for 12 h at 37°C, the optical density of the broth at 660 nm was 0.8 and 1.0, respectively. Each point represents the mean ± s.d. of three experiments.

As the number of coatings increased, the duration of the antibacterial activity of the matrix lasted longer, which is well correlated with the conventional in-vitro release study of cefadroxil (Figure 1). However, it is interesting to note that the antibacterial activity of the matrix without coating lasted for more than a week, although in-vitro release of cefadroxil was complete within 48 h (Figure 1). Compared with the 3 coatings, the diameter of the inhibition zone after 10 coatings was smaller but maintained a constant size for a longer duration, probably due to the zero-order release of cefadroxil from the matrix. The duration of antibacterial activity of matrices against *E.coli* was relatively shorter than that against *S. aureus*. Since cefadroxil is a first-generation antibiotic, it should be less active against Gram-negative *E. coli* than against Gram-positive *S. aureus*.

Broth method

Figure 5 shows the duration of antibacterial activity of polyurethane matrices, determined using broth, against *S. aureus* and *E. coli*. The optical density of the broth at 660 nm, after inoculating *S. aureus* or *E. coli* without cefadroxil and incubating for 12 h at 37 °C, was 0.8 and 1.0, respectively. Thus, the matrices that resulted in

optical density higher than these values were considered to have no antibacterial activity. Generally, the duration of antibacterial activity of polyurethane matrices determined using the broth and the agar showed similar patterns. For example, as the number of coatings increased, the duration of antibacterial activity of the matrices against *S. aureus* increased up to 30 days (Figure 5A). The antibacterial activity of uncoated matrices against both *S. aureus* and *E. coli* also lasted for longer than one week in the broth study. However, the duration of antibacterial activity using the broth was relatively longer than that observed in the agar study. This is probably because the release of cefadroxil into the broth (solution state) was easier than into the agar (solid state).

Moreover, although in-vitro release of cefadroxil from the coated matrix continued for more than 30 days (Figure 1), the duration of the antibacterial activity of the matrices after coating was less than 30 days in both agar and broth studies. This might be due to the loss of antibacterial activity of cefadroxil during storage. In both agar and broth studies, the antibacterial activity of the matrix without coating lasted for more than a week, although in-vitro release of cefadroxil completed within 48 h. Trace amount of antibiotic on the surface of the device after burst release might be enough to maintain the antibacterial activity of the matrix for even a week. Thus, the conventional in-vitro release study does not correlate well with the antibacterial activity of the device itself.

In-vivo antibacterial activity of matrices

Figure 6 shows the duration of antibacterial activity of the polyurethane matrices when implanted in the subcutaneous space of the dorsal region in rats. After removing each matrix at predetermined time intervals, the diameter of the inhibition zone was measured using agar, as described above. The duration of antibacterial activity of the matrices was well correlated with that of the agar study (Figure 4). Polyurethane matrix without coating inhibited the growth of bacteria for one week when implanted in rats. As the number of coatings increased, the polyurethane matrices showed antibacterial activity for a longer duration in-vivo.

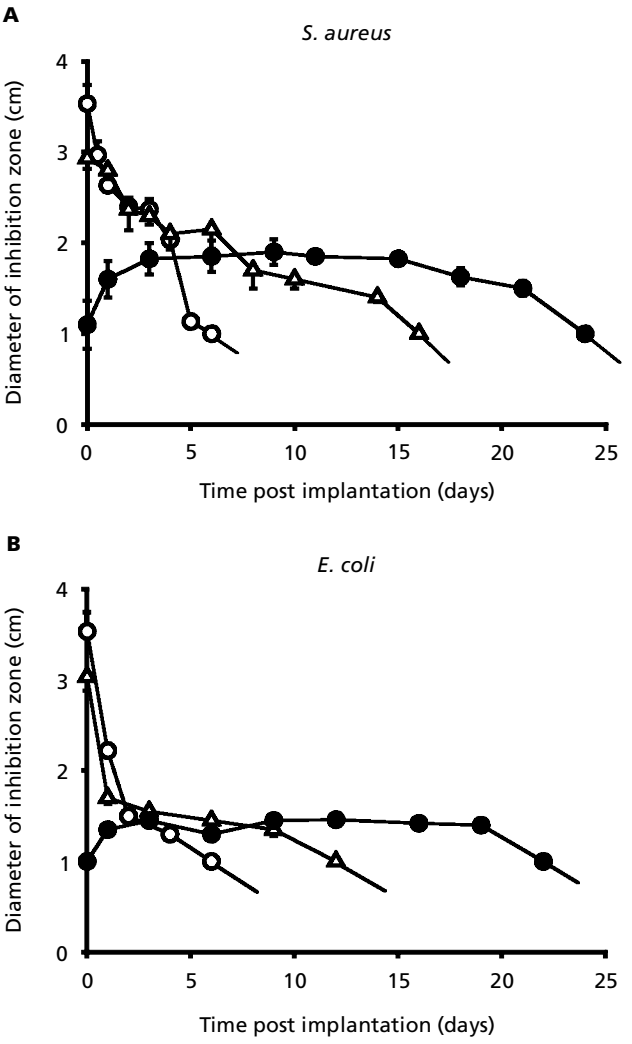

Figure 6 Duration of antibacterial activity of polyurethane matrices determined in-vivo. Matrices were implanted in the subcutaneous space of rat dorsal region, and then removed at predetermined time intervals. Matrices contained 40% lyophilized powder (particle size, 62–88 μm; pore former–cefadroxil, 1:1) without coating (○) or with 3 coatings (△) or 10 coatings (●) produced by dipping in polyurethane–THF (1:40 w/w) solution. The antibacterial activity of the matrices against *S. aureus* (A) and *E. coli* (B) was determined from the diameter of the inhibition zone by using agar. Each point represents the mean ± s.d. of four matrices.

Conclusions

The duration of antibacterial activity of polyurethane matrix was successfully determined in-vitro by transferring the matrix to a fresh culture medium. In-vivo evaluation was also feasible by implanting the matrices in rats, and then measuring the antibacterial activity of the matrix at predetermined time intervals. These modified in-vitro and in-vivo methods seem to be suitable for evaluating the duration of antibacterial activity of polyurethane matrices more accurately than the conventional in-vitro release study. These methods could be applied to determine the duration of antibacterial activity of any biomaterial itself or of systems designed for the local delivery of antibiotics.

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