Adsorption and release of antibiotics from morselized cancellous bone

In vitro studies of 8 antibiotics

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We studied the basic release patterns of antibiotics from cancellous bone in vitro. Antibiotic-impregnated bone was compressed into a wire-mesh cylinder and the release of antibiotic was assessed by two different in vitro methods: agar diffusion and broth elution. The zones of inhibition were measured on seeded agar and the amounts of antibiotics released in elution tubes were assessed by a bioassay. The study continued for 21 days with daily transfer of the cylinders. The results indicated that benzylpenicillin, dicloxacillin, cephalotin, netilmicin, clindamycin, vancomycin, ciprofloxacin and rifampicin were adsorbed to cancellous bone in vitro. Compared to broth elution, agar diffusion showed a prolonged period of release, owing to the small amounts of antibiotic leaking out of the cylinder into the agar. The betalactams had antibacterial activity in broth for a shorter time than the other antibiotics. The release patterns of the betalactams were similar, in spite of their differences in thermal stability. Only rifampicin showed a concentration higher than MIC for longer than 21 days.

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Submitted 98-08-22. Accepted 99-03-06

A few years after penicillin became available for clinical use, De Grood (1947) mixed it with cancellous bone when filling bone defects. Since then, only a few in vitro and in vivo studies of antibiotic-impregnated bone have been published (McLaren and Miniaci 1986, Miclau et al. 1993). Two clinical studies reported a mixture of antibiotics with cancellous bone (McLaren 1988, Perry 1996). The incorporation of an antibiotic in cancellous bone does not seem to influence the healing characteristics of the bone (Lindsey et al. 1993). We studied the adsorption of various types of antibiotics into morselized cancellous bone and evaluated the basic patterns of release in vitro.

Material and methods

Human cancellous bone allograft was prepared by passing it through a bone mill (Aesculap, Coarse, HARRIS, GB 44) under sterile conditions. 15 g of morselized bone was placed in a small metal bowl containing 20 mL of antibiotic solution. 8 different commercially available antibiotics were used (Table). After 10 minutes at room temperature, the fluid was cleared from the morselized bone by straining it through gauze. The gauze was twisted to leave a lump of almost completely dry bone. This was compressed into a wire-mesh cylinder (diameter 11 mm, height 10 mm) made of stainless steel. Each cylinder had a wire-mesh bottom.

The mean weight of bone in each cylinder was 1.0 (0.9–1.1) g. The cylinders to be used on the agar surface had three small pins in the bottom to

8 antibiotics were used for impregnation of morselized human cancellous bone allograft

	mg/mL
Benzylpenicillin (Penicillin, A.L.)	100
Dicloxacillin (Diclocil, Bristol-Meyers Squibb)	100
Cephalotin (Keflin, Lilly)	100
Netilmicin (Netilyn, Schering-Plough)	100
Vancomycin (Vancocin, Lilly)	50
Ciprofloxacin (Ciproxin, Bayer)	2
Clindamycin (Dalacin, Upjohn)	150
Rifampicin (Rimactan, CIBA)	60

Figure 1. Wire-mesh cylinders were made of stainless steel. The wire-mesh cylinder used on the agar surface (left) had three small pins to maintain a fixed position on the agar. Antibiotic-impregnated bone was compressed into the cylinders.

Figure 2. The zones of inhibition (diameter) were measured after incubation of the cylinders on Mueller Hinton medium, floated with *S. aureus* ATCC 25923 suspension at 37 °C for 24 h.

keep them in a fixed position on the agar (Figure 1). Antibiotic-impregnated bone was analyzed in triplicate in agar diffusion and broth elution experiments. Bone impregnated with saline but no antibiotic was used as a control. *Staphylococcus aureus* ATCC 25923 was used in bioassay to monitor antibiotic release. To ensure a standardized bacterial inoculum throughout the experiments, an overnight culture of *S. aureus* ATCC 25923 on blood agar plate was suspended in Mueller Hinton broth (Difco Laboratories, Detroit, MI, USA) and frozen in small aliquots at –80 °C. After thawing, the viable bacterial count was 1×10^9 Colony Forming Units (CFU)/mL with no loss of viability in the frozen state during the experiment.

Aliquots were thawed daily immediately prior to use and the concentration adjusted to the appropriate level (see below). The susceptibility of S.aureus ATCC 25923 to the antibiotics studied was determined by the E-test, according to the instructions of the manufacturer (AB BIODISK, Solna, Sweden), and the Minimum Inhibitory Concentration (MIC) was read (benzylpenicillin 0.047 mg/L, dicloxacillin 0.19mg/L, cefalotin 0.25 mg/L, netilmicin 0.25 mg/L, vancomycin 1.5 mg/L, ciprofloxacin 0.5 mg/L, clindamycin 0.094 mg/L, rifampicin 0.016 mg/L). Two in vitro methods were used: agar diffusion and broth elution.

Antibiotic release assessed by agar diffusion

A Petri dish (diameter 14 cm) with a standardized amount (90 mL) of Mueller Hinton Medium (broth + agar) was floated with 5 mL of a *S. aureus* ATCC 25923 suspension (2×10^5 CFU/mL). Superfluous bacterial suspension was removed and the plate was dried. This procedure consistently yielded semiconfluent bacterial growth. Cylinders with bone in triplicate of each antibiotic were placed on one plate and incubated at 37 °C for 24 h. The height of the cylinders enabled the lid of the plates to lie just on the cylinders.The zones of inhibition (diameter) were measured and the cylinders were transferred to a new seeded plate and incubated (Figure 2). The study continued for 21 days, with daily transfer of the cylinders on seeded agar.

Antibiotic release assessed by broth elution

The cylinder with its content was put into an elution tube (15 × 100 mm sterile glass tube), containing 5 mL of cation-adjusted Mueller Hinton broth and incubated at 37 °C for 24 h (Figure 3). This cylinder was then lifted from the test tube and fluid was removed by pressing the cylinder

Figure 3. The antibiotics were eluted in Mueller Hinton broth at 37 °C by daily transfer of the cylinders into 5 mL of broth. The release of antibiotics into the broth was determined with a bioassay, using *S. aureus* ATCC 25923 as an inoculum.

against the inner wall of the tube. It was washed by submerging it into 5 mL of 0.9% NaCl and it was then put into a new elution tube with 5 mL of Mueller Hinton broth. Preliminary studies did not show any differences on the subsequent antibiotic release between one, two, three or four washing procedures. The release of antibiotic into the elution tube was determined by a bioassay, employing a modification of broth microdilution for MIC determination (Tamashiro 1994). Briefly, 2 mL broth from the elution tube was diluted two-fold serially in Mueller Hinton broth. As inoculum, 5×10^5 CFU/mL (final concentration) of *S. aureus* ATCC 25923 was used. After incubation at 37 °C for 24 h, the highest dilution with no visible growth was recorded. This should represent an antibiotic concentration equal to or higher than the MIC. A minimum estimate of the concentration of antibiotic in the elution tube could then be made by multiplying the dilution factor of the last tube with no visible growth by the MIC. In this study, results are expressed as "Times above MIC", i.e., the dilution factor. The study was continued for 21 days by daily transfer of the cylinders into 5 mL of elution broth. On each day, the first tube of the dilution series showing visible growth was cultured on blood agar to ensure that the growth was due to *S. aureus* ATCC 25923 and not due to bacterial contamination.

Stability of the antibiotics at 37 °C

We aimed to study the influence of the thermal stability of the antibiotics at 37 °C. The various antibiotic solutions were kept at 37 °C. On days 0, 1, 3, 10 and 21, the antibacterial activity against *S. aureus* ATCC 25923 for each antibiotic was determined with the bioassay, as described above. Results are expressed as "Times above MIC".

Results

The control cylinder showed no inhibitory bacterial effect. Figure 4 shows the zones of inhibition (diameter) on agar for each antibiotic. The betalactams—i.e., benzylpenicillin, dicloxacillin and cefalotin—all showed a steady decline from around a 50 mm zone of inhibition. Benzylpenicillin and dicloxacillin reached 11 mm on day 19. For these two antibiotics, there was an unexplained divergence of the three parallels from day 11 on. The three parallels of cefalotin still had a small zone of inhibition on day 21. Netilmicin, clindamycin and rifampicin showed patterns of almost constant release with large inhibition zones throughout the study period. Ciprofloxacin showed a zone of 48 mm (46–49) on day 1, decreasing to 24 mm (21–25) on day 2 and from there on showing a steady state curve around an 18 mm zone of inhibition.The inhibition zone of vancomycin showed a steady decline until it reached 11 mm on day 12. Due to a technical failure, one of the three parallels of vancomycin was excluded.

Figure 5 shows the amount of antibiotic eluted in 5 mL broth expressed as "Times above MIC" (TAM). For all the antibiotics, the three parallels showed almost the same dilution factor, never diverging more than ± one step. The mean values are listed. The betalactams—benzylpenicillin, dicloxacillin and cefalotin—showed a rapid decline from very high values on day 1 (16,000–130,000 TAM) to zero on day 7. Netilmicin, vancomycin, ciprofloxacin and clindamycin all had a less steep curve and around day 14, there was no bacteriostatic effect in the broth. Only rifampicin showed a concentration in the broth higher than MIC for longer than 21 days.

Figure 6 shows the stability at 37 °C for each type of antibiotic. The betalactams were less stable than the other antibiotics. However, there were significant differences between the three betalactams concerning thermal stability: benzylpenicillin had a 700,000-fold reduction in activity from day 1 to day 21, dicloxacillin a 2,000-fold reduction and cefalotin a 50-fold reduction. The activities of netilmicin, vancomycin, clindamycin, ciprofloxacin and rifampicin were unchanged after 21 days.

Discussion

Our study showed that benzylpenicillin, dicloxacillin, cefalotin, netilmicin, vancomycin, ciprofloxacin, clindamycin and rifampicin were adsorbed into morselized cancellous bone. Morselized cancellous bone acted well as a vehi-

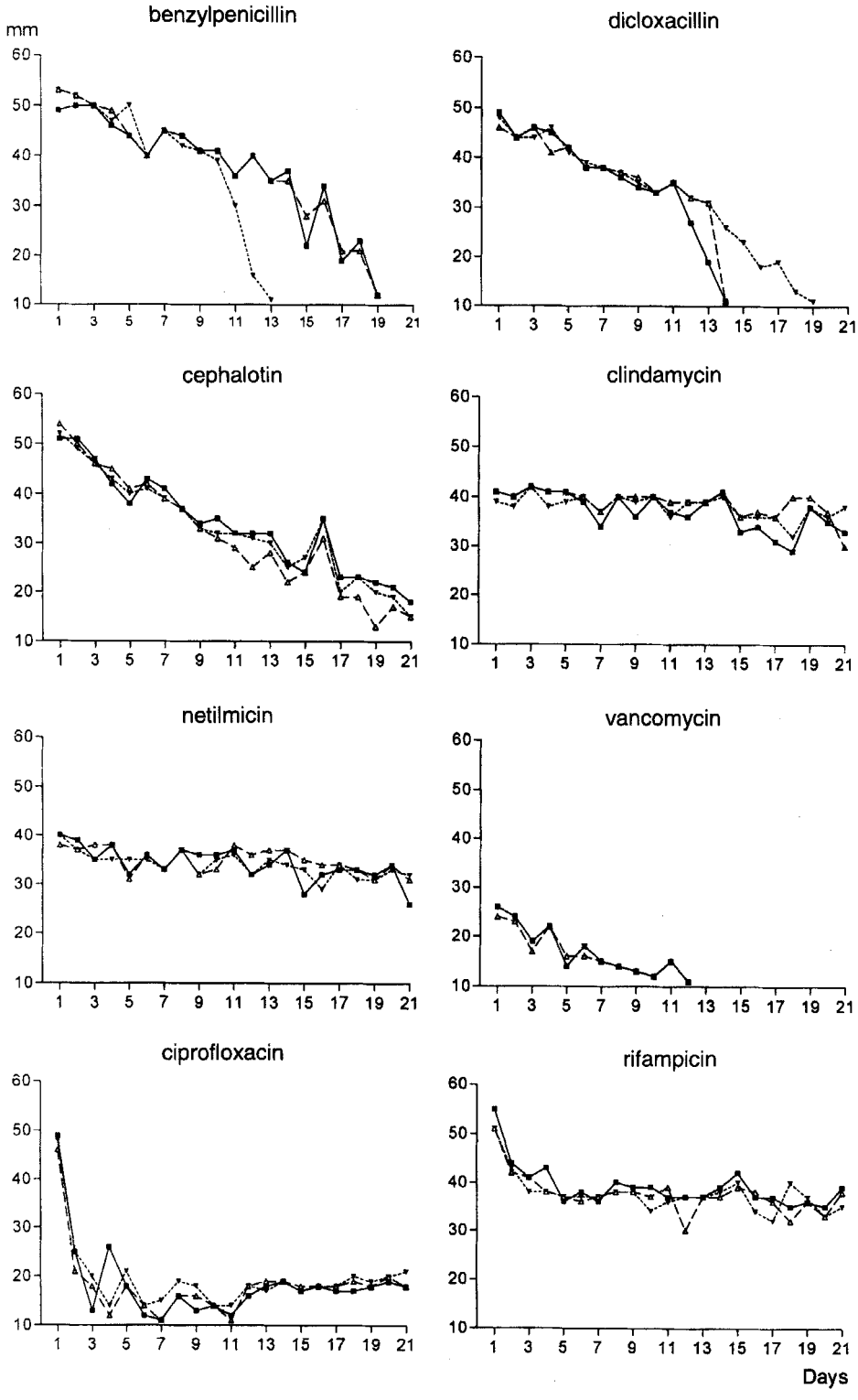

Figure 4. Antibiotic release, assessed by agar diffusion. A zone of 11 mm is equal to the diameter of the cylinder, i.e., no inhibition.

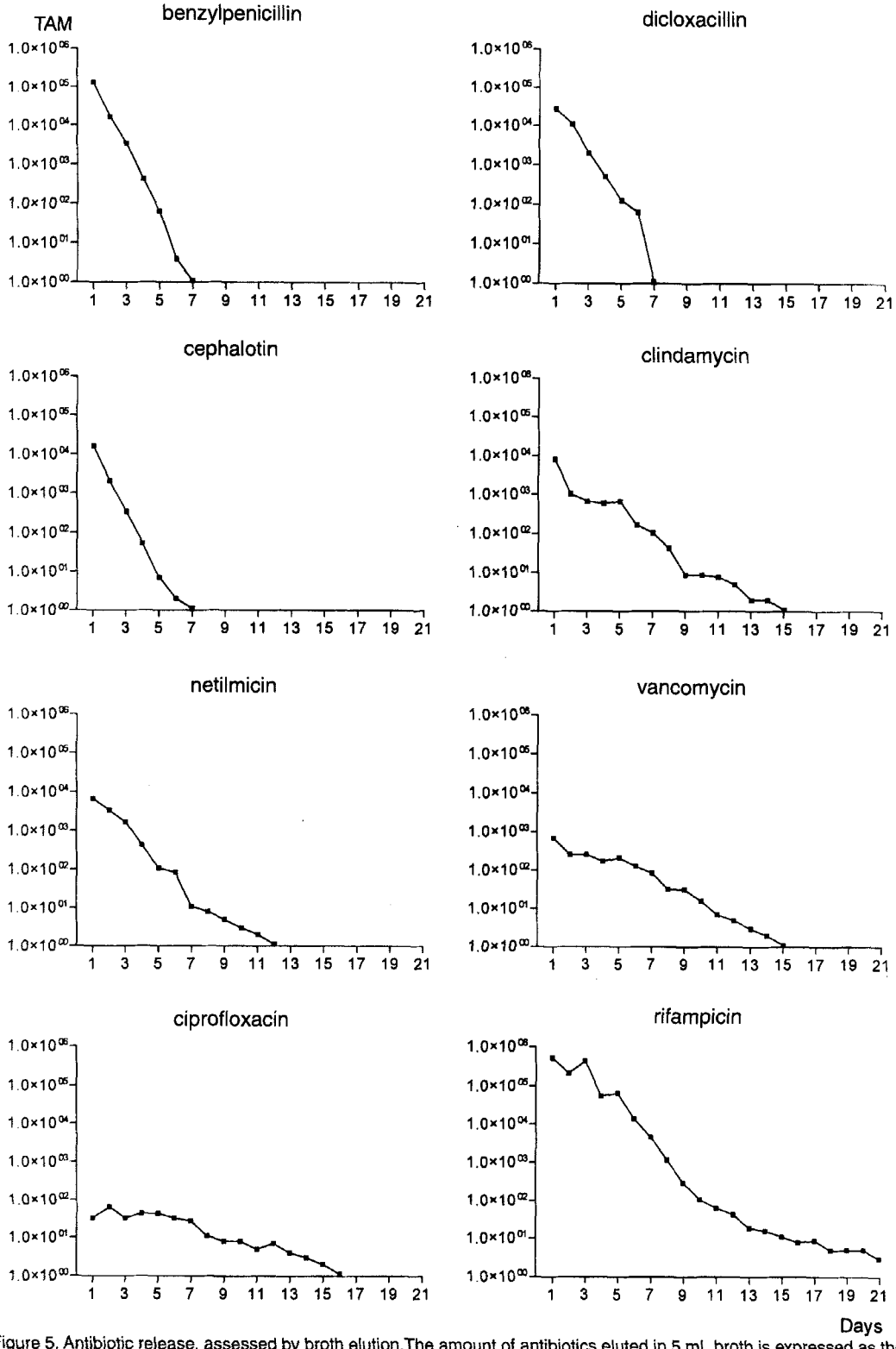

Figure 5. Antibiotic release, assessed by broth elution.The amount of antibiotics eluted in 5 mL broth is expressed as the dilution factor. Times above MIC (TAM), on a semilogarithmic scale.

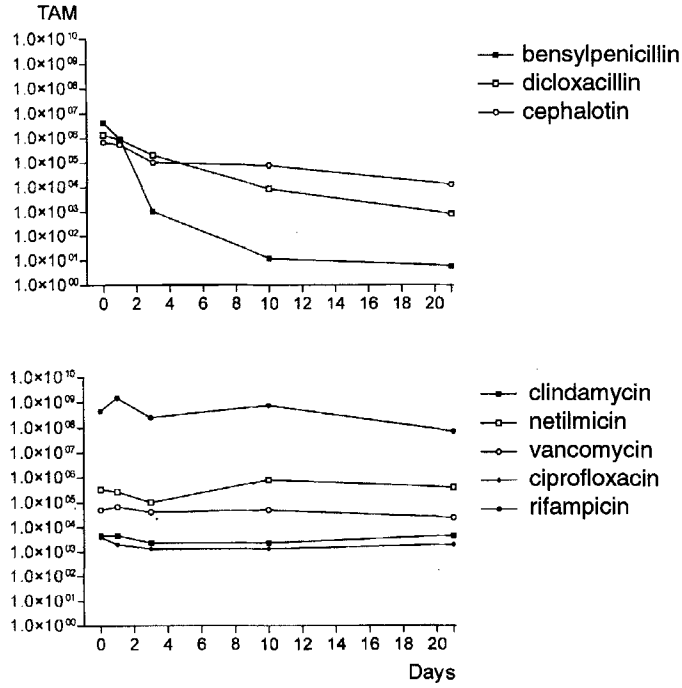

Figure 6. The thermal stability of the antibiotics was measured. Using a modification of broth microdilution for MIC determination, the activity against *S. aureus* ATCC 23 was measured on days 0, 1, 3, 10 and 21. The remaining concentration is expressed as dilution factor. Times above MIC (TAM) on a semilogarithmic scale.

cle for prolonged antibiotic delivery in vitro. The method used to study release of antibiotics from morselized cancellous bone, using a wire-mesh cylinder containing antibiotic-impregnated bone on agar or in broth, seems to be a reliable in vitro method. Particularly broth elution displayed figures with minimal variation between the three parallels.

Agar diffusion showed a longer period of release than broth elution. This is a reflection of the very small amounts of antibiotics leaking out of the cylinder into the agar. Preliminary studies have shown that, by pressing the cylinder deeper into the agar, the zone of inhibition is enlarged, reflecting more antibiotic being released from the cylinder.

In this study, agar diffusion apparently reflects the thermal stability of the various antibiotics, with the exception of vancomycin.

The release pattern of the 8 antibiotics using broth elution resembled the curve of a negative exponential function. This is expressed as a straight line on a semilogarithmic scale, indicating that the amount of antibiotic released each day was proportional to the amount of residual antibiotic left in the bone. A disadvantage of our method, however, is that we could not calculate the ratio of antibiotic released vs. the amount of antibiotic present in the bone. In accordance with their thermal stability, the betalactams had antibacterial activity in broth for a shorter time than the other five antibiotics. However, the three betalactams showed similar elution characteristics, in spite of their significant differences in thermal stability. Cefalotin thus is relatively stable per se, but is rapidly eluted from bone.

In vitro studies of local antibiotic release have employed various microbiological methods. Measurement of inhibition zones on seeded agar is the same technique as used in studies of antibiotic release from polymethylmethacrylate (PMMA) bone cement (Walenkamp 1983). Walenkamp states that agar diffusion is a qualitative assay to determine which antibiotic will be released from bone cement, or in this case, morselized cancellous bone. With respect to broth elution, we used 5 mL as an elution volume. Other investigators have used other volumes to study release of anti-

biotics from PMMA (Adams et al. 1992, Penner et al. 1996). Only studies comparing an in vitro to an in vivo method can indicate the appropriate elution volume.

In this study, we used a bioassay to evaluate the antibiotic released, in contrast to exact quantifications of antibiotic concentration. The main advantage of a bioassay is that the effective inhibitory concentration of the antibiotic is registered, including antibiotics, which otherwise are not readily subject to quantification of concentration.

The use of antibiotic-impregnated PMMA bone cement and beads have proved to be effective prophylactic and treatment options (Buchholz et al. 1981, Calhoun and Mader 1989, Espehaug et al. 1997). However, polymerization of methylmethacrylate is an exothermic reaction and the heat released by polymerization may lead to necrosis of the bone. In vitro studies of PMMA beads have shown that only 10–20% of the antibiotics mixed with PMMA is released (Picknell et al. 1977, Miclau et al. 1993). Other delivery systems with biodegradable vehicles have therefore been studied (Becker et al. 1994, Mousset et al. 1995, Nie et al. 1995, Humphrey et al. 1998).

We conclude that morselized cancellous bone serves well as a vehicle for adsorption and subsequent release of benzylpenicillin, dicloxacillin, cefalotin, netilmicin, vancomycin, ciprofloxacin, clindamycin and rifampicin. The release patterns vary of these antibiotics. Agar diffusion reflects the stability of the antibiotics, while broth elution probably more reliably indicates differences in release characteristics.

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