

The Elution of Colistimethate Sodium from Polymethylmethacrylate and Calcium Phosphate Cement Beads

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Abstract

Gram-negative bacilli resistance to all antibiotics, except for colistimethate sodium (CMS), is an emerging health-care concern. Incorporating CMS into orthopedic cement to treat bone and soft-tissue infections due to these bacteria is attractive, but the data regarding the elution of CMS from cement are conflicting. The *in vitro* analysis of the elution of CMS from polymethylmethacrylate (PMMA) and calcium phosphate (CP) cement beads is reported. PMMA and CP beads containing CMS were incubated in phosphate-buffered saline and the eluate sampled at sequential time points. The inhibition of the growth of a strain of *Acinetobacter baumannii* complex by the eluate was measured by disk diffusion and microbroth dilution assays, and the presence of CMS in the eluate was measured by mass spectroscopy.

Bacterial growth was inhibited by the eluate from both PMMA and CP beads. Mass spectroscopy demonstrated greater elution of CMS from CP beads than PMMA beads. The dose of CMS in PMMA beads was limited by failure of bead integrity.

CMS elutes from both CP and PMMA beads in amounts sufficient to inhibit bacterial growth *in vitro*. The clinical implications of these findings require further study.

Gram-negative bacilli resistant to multiple antibiotics have emerged as a global healthcare concern.¹ *Acinetobacter baumannii* complex (ABC) is an example of a gram-negative bacilli with a predilection to acquire resistance to multiple antibiot-

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ics. Strains which are resistant to all antibiotics except colistimethate sodium (CMS) are increasingly reported.² Although usually considered a nosocomial pathogen associated with central-line associated bacteremia and ventilator-associated pneumonia, ABC has been reported as causing trauma-related osteomyelitis and soft-tissue infections.³ Cement beads impregnated with antibiotics have been used in the treatment of bone/wound infection as a method to deliver antibiotics directly to the site of infection.⁴ Incorporating CMS into cement beads to treat multi-drug resistant ABC infections is theoretically attractive, as high doses could be delivered directly to the site of infection and systemic CMS has known toxicities.⁵ However, the data supporting the use of CMS-containing orthopedic beads are sparse and conflicting, with 1 study demonstrating that CMS failed to elute from Palacos® (polymethylmethacrylate [PMMA]) cement in clinically meaningful quantities, while 2 others report *in vitro* activity when colistin, along with erythromycin, was added to Simplex P bone cement and when colistin sulfate was added to PMMA beads; CMS was not studied.⁶⁻⁸ Before CMS-containing cement can be used confidently in the clinical arena, whether the antibiotic elutes from cement needs to be evaluated. We sought to answer that question and report the elution of CMS, from both PMMA and calcium phosphate (CP) beads, as measured by 3 separate laboratory assays.

MATERIALS AND METHODS

Antibiotic-Containing Cement Beads

Commercially available kits of PMMA (Stryker Howmedica Osteonics, Mahwah, New Jersey) or CP (Wright OsteoSet, Arlington, Tennessee) cement were used to make beads. Separate batches of cement containing 150 mg (1 vial), 300 mg (2 vials), 450 mg (3 vials), 600 mg (4 vials), or 750 mg (5 vials) of CMS (Colistimethate Monarch Pharmaceuticals Bristol, Tennessee and X-Gen Pharmaceuticals Northport, New York) were made. Beads of approximately 6 mm in diameter were created using molds supplied in the PMMA kit. Positive control beads lacking CMS were made by adding 2.4 g of tobramycin (X-Gen) to each batch of PMMA or CP, and negative control beads were made without incorporating CMS or tobramycin.

Elution Protocol

Three beads were submerged in 5 mL of phosphate saline buffer (PBS, pH 7.4) in polystyrene tubes. The tubes were incubated in ambient air at 37°C while gently rotating at 200 rpm on a mechanical shaker (Barnstead/Lab-line MaxQ 2000). Three hundred and seventy-five μL were aspirated from each tube at 6 and 24 hours, and 3, 5, 7, 14, 21, 31, 42, and 62 days. The eluate in the tubes was not replenished. Tube agitation was stopped for 45 minutes prior to pipetting to prevent pieces of degraded cement beads from being inadvertently aspirated.

Bacteria

A colony of *A baumannii* with a minimal inhibitory concentration (MIC) of less than 2 mcg/mL for tobramycin and colistin sulfate was obtained from the hospital clinical laboratory. For the assays, a 0.5 MacFarland (approximately 1.0×10^8 colony-forming unit/mL) suspension was grown in Cation Adjusted Mueller Hinton Broth.

Disk Diffusion Assay

Twenty-five microliters of eluate was placed onto a 0.6 cm blank paper disk (Becton Dickinson, Franklin Lakes, New Jersey). A 0.5 MacFarland bacterial suspension of *A baumannii* was inoculated on a large Mueller Hinton Agar plate for confluent growth and then the disk was positioned and incubated in ambient air at 37°C for 18-24 hours. The zone of inhibition (ie, the diameter of the zone without growth) was measured. Each assay was performed in triplicate and the median zone of inhibition at each eluate concentration and time-point was calculated.

Microbroth Dilution Assay

One hundred microliters of eluate was serially diluted with a 0.5 MacFarland suspension of *A baumannii* in a 96 well plate. The plates were incubated in ambient air at 37°C for 18-24 hours and the lowest dilution of eluate, which completely inhibited bacterial growth as detected by the unaided eye, was recorded. Each assay was performed in triplicate, and the median dilution of eluate, which inhibited bacterial growth, was calculated.

Mass Spectrometry

Eluate samples were analyzed for colistin A and B with a reversed phase high performance liquid chromatography (LC) mass spectrometry³ (MS³) procedure. The peak area ratios (PARs) of colistin A and colistin B, to the internal standard leucine enkephalin was determined by extracting the ion produced in MS³. The PARs of the standard curve samples were fit by equally weighted least squares linear regression. All data processing was performed using the Xcalibur Quan Browser Software (Thermo Scientific, Waltham, Massachusetts).

Since colistin A and B are not available as purified standards, a commercial preparation of colistin sulfate was obtained (Sigma-Aldrich, St. Louis, Missouri).

The triply charged ions yielded the highest signal and were selected for LC-MS³ analysis.⁹ The proportion of colistin A and B was calculated as the ratio of their respective peak areas to the summed peak area of both. The assumption that the commercial preparation is composed only of colistin A and B likely results in overestimation since polymyxins other than A and B are likely present.¹⁰ The percentage of colistin A and B were found to be $63.8 \pm 2.2\%$ and $36.1 \pm 1.2\%$, respectively, and was used to prepare a standard curve.

Eluate samples were obtained at day 62 from PMMA and CP beads made from batches containing 150 mg and 300 mg of CMS and analyzed for colistin A and B. Samples were serially diluted from 200-20,000 with 11% formic acid and analyzed with the LC-MS³ procedure described above. Colistin A and B are not available as purified standards. A commercial preparation of colistin sulfate was obtained (Sigma-Aldrich) and a 10 $\mu\text{g}/\text{mL}$ solution in 50% acetonitrile, 0.1% formic acid was prepared. Direct injection into the mass spectrometer yielded single, double, triple, and quadruple charged ions of colistin A and B with and without H_2SO_4 adducts. The triply charged ions yielded the highest signal and were selected for LC/MS³ analysis.

Statistical Methods

The effects of bead, drug, and time were examined using analysis of variance. Bonferroni corrected *P* values are reported for pair-wise comparisons. For microbroth dilution, inhibition levels at each time point were compared between concentrations using the Wilcoxon signed ranks test.

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RESULTS

PMMA Beads

With PMMA beads, the dose of CMS incorporated into each batch of cement was limited to 150 mg and 300 mg, as higher doses resulted in loss of bead integrity. As measured by the disk diffusion (DD) assay, CMS eluted in concentrations sufficient to inhibit the growth of ABC, with 300 mg having a larger zone of inhibition than 150 mg ($P < .0005$). The Table displays the median zones of inhibition for the various concentrations at all time points. The zone of inhibition was not statistically different from 6 hours to 62 days after bead placement (Figure). Bacterial growth was also inhibited as measured by the microbroth dilution (MBD) assay, although there was

Table. Bacterial Inhibition and Elution of Various Doses of CMS When Incorporated Into PMMA and CP Cement Beads.

Cement Type	Amount of CMS (mg)*	Median Zone of Inhibition on Disk Diffusion Assay (mm)†	Median Dilution Bacterial Growth Observed‡	Colistin A (mean ug/ml)§	Colistin B (mean ug/ml)§
PMMA	150	9	1:100	47.4	20.7
PMMA	300	11	1:100	63.9	28.9
CP	150	18	1:1000	1700	670
CP	300	18.5	1:100000	4000	1730
CP	450	19	1:1000	Not performed	Not performed
CP	600	20	1:1000	Not performed	Not performed
CP	750	20	1:1000	Not performed	Not performed

*Doses of greater than 300 mg of CMS per batch of PMMA were not tested due to bead instability

†Median zone of inhibition as measured at all time points

‡Median dilution as measured at all time points

§Eluent from day 62, as measured by mass spectroscopy

Abbreviations. CMS, colistimethate sodium; CP, calcium phosphate; PMMA, polymethylmethacrylate.

no measurable difference in bacterial inhibition between the 150 mg and 300 mg doses (Table) or over the various time-points (data not shown). Mass spectroscopy detected colistin A and colistin B in the eluate sampled at the day 62 time-point, with more colistin detected in the 300 mg concentration than the 150 mg concentration (Table).

Eluate from the negative control beads (lacking CMS) demonstrated no inhibition of bacterial growth. Eluate from the positive control beads containing tobramycin demonstrated a median zone of inhibition of 27 mm in the DD assay and inhibited bacterial growth when diluted to 1:100 in the MBD assay. There was no statistical change in bacterial inhibition over the various time points.

CP Cement Beads

CMS eluted from CP beads in concentrations sufficient to inhibit the growth of ABC as measured by both DD and MBD assays. The zone of inhibition increased slightly (2 mm) ($P<.0005$) with an increase in CMS from 150 mg to 600 mg per batch (Table). The zone of inhibition was not significantly different from 6 hours to 62 days after bead placement. The Figure displays the median zone of inhibition at different time points for the 150 mg and 300 mg concentrations. Due to space considerations, the other concentrations are not shown. In the MBD assay, the inhibition of growth increased one dilution from the 150 mg batch to the 750 mg batch (Table), and there was no change in inhibition when the eluate was sampled at sequential time-points (data not shown). Mass spectroscopy detected colistin A and colistin B in the eluate sampled at the day 62 time-point, with more colistin detected in the 300 mg concentration than the 150 mg concentration (Table).

Eluate from the negative control

beads (lacking CMS) demonstrated no inhibition of bacterial growth. Eluate from the positive control beads (containing tobramycin) demonstrated a median zone of inhibition of 30 mm in the DD assay and inhibited bacterial growth when diluted to 1:100 in the MBD assay. There was no statistical change in bacterial inhibition over the various time points.

DISCUSSION

In this study, as measured by 3 different assays (micro-broth dilution, disk diffusion, and mass spectrometry), CMS was demonstrated to elute from both CP and PMMA cement. When incorporated into CP cement, CMS elutes at higher concentrations and provides greater bacterial inhibition than when incorporated into PMMA cement. Antibiotic elution from both cements reached a plateau relatively quickly and did not change significantly over time. Although increasing the amount of CMS into the cement mixtures resulted in eluates with more detectable colistin, the integrity of PMMA beads was compromised beyond a dose of 300 mg of CMS per batch of cement.

The methodology for the bioassays used in this study were developed based on prior reports and there is no

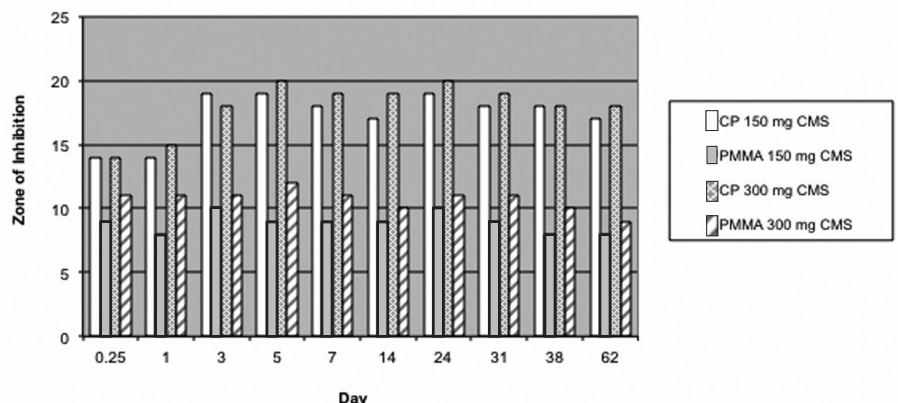


Figure. Zones of inhibition over time from eluates of calcium phosphate and polymethylmethacrylate beads containing 150 or 300 mg of colistimethate sodium per batch.

single standardized methodology for the detection of anti-bacterial activity in antibiotic-impregnated cement beads.¹¹⁻¹⁸ The decision to incorporate CMS, rather than colistin sulfate, into cement beads for this study was made because colistin sulfate is only FDA-approved for topical and oral use, and thus, presumably, CMS would be used to manufacture antibiotic-impregnated beads.

An important limitation to our findings is that the Clinical and Laboratory Standards Institute (CLSI) has not published breakpoints defining the activity of CMS against *A baumannii*, and thus, the inhibition of bacterial growth observed in our assays may not translate into clinical efficacy. We have demonstrated that CMS elutes from both CP and PMMA beads in vitro, and the next step in evaluating this therapy would be to perform in vivo studies. Crane and colleagues⁸ recently reported that colistin-sulfate elutes from PMMA beads and reduced the development of osteomyelitis in a murine challenge model. However, this study used colistin-sulfate, not CMS, and may not be reflective of the type of colistin likely to be used in humans. In addition, the study reported the prevention of osteomyelitis, and thus, the efficacy of this intervention for treating established infection is unknown. At this point, the efficacy and potential local and systemic toxicities associated with colistin-bead use in humans are undefined. A recent study reported that several antibiotics, including CMS, inhibited cell viability and osteogenic activity in a laboratory model, a finding which highlights the potential multifactorial effects associated with the local delivery of antibiotics.¹⁹ Until further studies are performed to better define the risks associated with the administration of CMS incorporated into cement, we suggest that this intervention be reserved as a measure of last resort for specific patient populations, such as those known to be infected with bacteria susceptible only to CMS. Given the dearth of antibiotics with activity against multi-drug resistant gram-negative bacilli, novel delivery systems to maximize the efficacy of our existing agents are urgently needed. We look forward to studies defining the clinical role of CMS-impregnated cement in the treatment of infections caused by these bacteria.

AUTHORS' DISCLOSURE STATEMENT

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