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Analysis of linezolid and tigecycline as candidates for local prophylaxis via antibiotic-loaded bone cement

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Running Title: Analysis of linezolid and tigecycline in bone cement

Objective: To assess the use of Gram-positive specific antibiotic linezolid and the broad-spectrum antibiotic tigecycline, for use in local antibiotic delivery via antibiotic-loaded bone cement.

Methods: Linezolid and tigecycline were added to Biomet bone cement at varying concentrations. Antibiotic elution over one week was quantified by HPLC-MS. The effect of wear on elution over 48 h was determined using a modified TE-66 wear tester. Eluted antibiotics were used to determine MIC against a panel of clinically relevant bacteria. Impact

strength of antibiotic-loaded samples was determined using a Charpy-type impact testing apparatus. Cytotoxicity of eluted antibiotics against MG-63 cells was evaluated using an MTT assay.

Results: Linezolid and tigecycline eluted from bone cement to clinically relevant levels within 1 hour and retained activity over 1 week. Mechanical wear significantly reduced elution of tigecycline but had little effect on elution of linezolid. Linezolid showed low cytotoxicity towards MG-63 cells with ≤ 300 mg/mL resulting in >50 % cell activity. Cytotoxicity of tigecycline was higher, with an IC_{50} of 5-10 mg/L.

Conclusions: Linezolid and tigecycline retain activity after elution from bone cement. The concentration of tigecycline may need to be carefully controlled due to cytotoxicity. The effect of wear on bone cement may need to be considered if tigecycline is to be used for local delivery. Up to 10% linezolid can be added without affecting the impact strength of the bone cement. These results are promising indications for future investigation of these antibiotics toward use in local antibiotic delivery strategies.

Introduction

Prosthetic joint infections present a rare but major complication in arthroplastic surgery. The incidence of infection across all arthroplastic procedures has been reported as ranging from 1 – 3%.[1-3] Revision surgery to remedy an infected joint prosthesis is associated with increased costs, longer stay in hospital and potential morbidity, compared to revision surgery after aseptic failure.[4-6] The number of arthroplastic procedures and the incidence of infection have increased over the last 10 years, as have the total costs associated with revision surgery.[4,5,7]

As the demand for arthroplastic surgery progressively rises, the costs associated with prosthetic joint infection are set to increase greatly. This has led to perioperative antibiotic prophylaxis strategies including the use of antibiotic-loaded bone cement becoming routine.[8,9]

The management of a prosthetic joint infection involves removal of the infected prosthesis and radical debridement of the surrounding infected tissue. This is followed by either a one-stage revision where a new prosthesis is implanted in a single procedure or a two-stage revision where a temporary spacer is used for several weeks before the new prosthesis is implanted. In both procedures antibiotic therapy is standard practice, commonly combining systemic antibiotic treatment with local delivery using antibiotic-loaded bone cement. Antibiotic-loaded cement is used to cement the prosthesis into place and, in the two-stage revision, is used to form the temporary spacer.[10]

Antibiotic-resistant organisms such as methicillin-, vancomycin- and multidrug resistant strains are increasingly becoming associated with failure of revision surgery. More than 50% of all prosthetic joint infections are caused by staphylococci such as *Staphylococcus aureus* and *Staphylococcus epidermidis* and it has been estimated that around half of all *S. aureus*-related periprosthetic joint infections are now methicillin resistant.[1,11-13] The ability of these organisms to acquire antibiotic resistance requires the use of new antibiotics to be explored for use in bone cement.

Here we evaluate linezolid and tigecycline for use in antibiotic-loaded bone cement systems and assess their suitability for this application. There are few studies investigating the inclusion of linezolid in bone cement[14,15] and, to our knowledge, there are no published data

on the inclusion of tigecycline in bone cement. Linezolid is a member of the oxazolidinone family of antibiotics and is active against most Gram positive organisms including many drug-resistant strains.[16] Tigecycline is a member of the glycylcycline family of antibiotics and has good activity against both Gram negative and Gram positive organisms.[17]

Materials and methods

Bacterial strains and growth conditions

All strains were maintained on Mueller-Hinton agar or Mueller-Hinton broth and grown overnight at 37°C. Clinical isolates of *S. aureus, S. epidermidis* and *Escherichia coli* were isolated from infected prostheses at the Northern General Hospital, Sheffield. *S. epidermidis* DSM 3269 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The *S. aureus* strain SH1000 was provided by Simon Foster, University of Sheffield.

Antimicrobial susceptibility

Serial dilutions of antibiotic standard solutions or serial dilutions of buffer from antibiotic elution experiments were prepared in triplicate with fresh Mueller Hinton broth in 96 microtitre well plates. Wells were inoculated with each microorganism in triplicate to a final density of 10^5 cfu/mL and incubated overnight at 37˚C. MICs were determined by eye and were defined as the lowest concentration of antibiotic that showed complete inhibition of growth.

MG63 cell culture

Cells were cultured on Eagles minimal essential medium (EMEM) containing 10 % fetal bovine serum (v/v), 2 mM glutamine and 1 % non-essential amino acids (v/v). Cells were incubated at 37˚C (5 % CO_2) and passaged three times a week.

MTT assay

MG63 cells were seeded at 2×10^3 cells per well in 100 µL of EMEM containing the appropriate concentration of antibiotic. Cells were incubated at 37˚C (5 % CO_2) for 48 h. After 48 h the medium was removed and fresh medium added. A 12 mM stock solution of MTT was prepared and 10 µL added to each well before incubating at 37˚C (5 % CO_2) for 4 h. An SDS-HCl (100 mg/mL, 0.01M HCl) stock solution was prepared and 100 µL added to each well before incubating for a further 4 h. Absorbance was measured at 570 nm and compared to positive control cultures containing no antibiotic.

Preparation of bone cement

Linezolid, tigecycline and gentamicin-containing bone cement samples were prepared by hand-mixing antibiotic powder (3% or 10% wt/wt) with Biomet Bone Cement R® powder until a homogenous mix was produced. The antibiotic cement powder was then mixed with the appropriate amount of polymethylmethacrylate (PMMA) monomer liquid in a Hi-Vac bone cement mixing bowl (Biomet) as per the manufacturer's instructions. Refobacin Bone Cement R® and Bone cement R (Biomet) were also prepared in a Hi-Vac bone cement mixing bowl (Biomet) as per the manufacturer's instructions. The bone cement was placed

into the relevant mould and allowed to cure for 1 hour. Once removed from the mould, antibiotic-loaded cement samples were stored at -20°C for up to 1 week until required in order to preserve antibiotic activity. The storage of bone cement at this temperature was shown to have no appreciable effect on elution of antibiotic (data not shown).

Static elution of antibiotic from bone cement samples

Antibiotic-loaded bone cement was placed in circular moulds and allowed to cure for 1 h to produce a 31 mm diameter x 7 mm thick disc. The resulting bone cement discs were then placed in 0.1 M ammonium acetate (pH 7.4) solution stirred at 300 rpm in a UV-opaque container and 0.5 mL aliquots of solution taken over 1 week and stored at -20 °C until analysed.

Evaluation of the effect of wear on antibiotic elution

Evaluation of the effect of wear on the rate of elution of antibiotics from the bone cement was carried out via a procedure based on that described by Dodds et al.,[18] as follows. The antibiotic-loaded bone cement was formed in an annulus-shaped mould and a 2 kg weight placed on top. The resulting annular samples were 40 mm outer diameter, 8 mm inner diameter and 10 mm thick. The sides of the annulus were coated with beeswax to ensure antibiotic could only elute from the outer perimeter. Controlled wear was generated by use of a HVOF-VPD hydroxyapatite (HA) coated 30 mm diameter x 3 mm thick Ti disc which was placed onto the lever arm specimen holder of a TE-66 microabrasive wear tester.[16] The sample was orientated so that the flat 10 mm thick outer perimeter was in contact with the HA-coated counter-face and a 2.5 N force exerted by the counter-face onto the outer

perimeter of the wearing cement sample. A container was placed beneath the assembly and filled with 0.1 M ammonium acetate solution (pH 7.4) until the lower portion of the cement sample was submerged. A magnetic stirrer was used to mix the solution in the container at 300 rpm and samples were rotated against the HA counter-face at 60 rpm for 51 h. The HA counter-face was repositioned every 10 - 12 h to ensure a sufficiently abrasive counter-face throughout the experiment. An extension shaft was fitted to the TE-66 to allow simultaneous rotation of an unworn control sample at the same speed. This sample was also partially submerged in a separate container filled with 0.1M ammonium acetate solution (pH 7.4). The experiment was placed in a UV-sealed air-tight container and the temperature and humidity constantly measured during the experiment. At regular intervals, 200 µL aliquots of solution were taken and stored at -20°C before analysis.

Quantification of antibiotics by LC-MS

Detection of linezolid was carried out on a Phenomenex Luna C_{18} reversed phase column (150 mm x 1 mm) attached to a Finnigan LCQ ESI-MS. The isocratic mobile phase was 0.1% aqueous trifluoroacetic acid (TFA)/acetonitrile (77:23) and the flow rate was 0.05 mL/min. Measurement of linezolid concentration was carried out by monitoring the protonated parent ion at m/z 338.2 and comparing the results to a standard curve. Quantification of tigecycline was carried out as described above except the isocratic mobile phase was 0.1% aqueous TFA/methanol (67:33) and monitoring the protonated parent ion at m/z 586.5.

Impact strength analysis

The impact testing was carried out as described by Barker et al.[19] using a Charpy-type impact tester (Hounsfield Plastics impact testing apparatus). Antibiotic-loaded bone cement was moulded into 44.45mm × 7.93mm × 7.93mm bars and notched using the Hounsfield notching machine (notch tip radius 0.25mm). Impact analysis was carried out according to BS ISO 179-1:2010 specifications[20] with the exception of the specimen dimensions. For each sample group 5 specimens were made and force applied to the un-notched side.

Statistical analysis

Statistical comparison of wear and non-wear samples was carried out by unpaired t-test. The statistical analysis of impact testing samples was carried out by one way analysis of variance. All statistical analysis was carried out using Microsoft Excel software

Results

Elution of antibiotic from bone cement

Elution of antibiotic from bone cement samples containing 3% (wt/wt) linezolid or 3% (wt/wt) tigecycline was monitored over a 1-week period. The concentration of linezolid eluted from the bone cement increased over the 1 week time period of the experiment (Fig 1). A maximum concentration of 12.2 ± 2.9 mg/L of linezolid was reached after 168 h and the initial elution rate of linezolid from bone cement was calculated as 213.4 ± 33.4 µg/hour/g bone cement. The concentration of eluted tigecycline initially increased to a maximum

198 concentration of 0.66 ± 0.35 mg/L after one hour and then decreased to 0.084 ± 0.025 mg/L
199 after 24 h and 0.014 mg/L ± 0.013 after 168 h (Fig 2). The initial elution rate of tigecycline
200 from bone cement was calculated as 32.8 ± 17.2 µg/hour/g bone cement.
201
202 **Effect of wear on elution of bone cement**
203
204 The results from three separate experiments to investigate the effect of wear on elution
205 behaviour of cement containing 3 % (wt/wt) tigecycline are shown in Fig. 3. The samples
206 were collected over a 51 h period and the maximum concentration of eluted antibiotic was
207 reached between 5 h and 12 h. The highest concentration overall was seen in the unworn
208 sample 2 after 12 h with a concentration of 2.1 mg/L compared to 0.1 mg/L in the worn
209 counterpart (Fig 3b). Although there is some variability in the maximum concentrations
210 between the three experiments, in all cases a clear trend can be seen with the elution from
211 unworn samples being significantly higher than the worn bone cement samples ($P < 0.05$).
212 After 1 hour the elution of tigecycline from unworn samples was 9.4 ± 2.6 µg/hour/cm^3
213 surface and the rate of elution from the worn samples was 2.3 ± 2.5 µg/hour/cm^3 surface.

214

215 The results from three separate experiments to investigate the effect of wear on elution
216 behaviour of cement containing 3 % (wt/wt) linezolid are shown in Fig. 4. The samples were
217 collected over a 51 h period and the maximum concentration of eluted antibiotic was reached
218 between 24 h and 51 h with concentration continuing to increase in all but one sample. The
219 highest concentration overall was seen in the worn sample 2 after 51 h with a concentration
220 of 53.1 mg/L (Fig 4b). No significant difference can be seen in the elution kinetics between
221 the worn and unworn linezolid samples ($P = 0.63$). After 1 hour the rate of elution from

unworn linezolid samples was 232.5 ± 22.4 µg/hour/cm^3 surface and the rate of elution from the worn linezolid samples was 242.4 ± 24.3µg/hour/cm^3 surface. The rates of antibiotic elution from both unworn and worn linezolid samples were > 100-fold higher than that of the worn tigecycline samples and 24.8 and 25.9-fold higher respectively than the unworn tigecycline samples.

Antimicrobial activity of eluted antibiotics

S. aureus (SH1000), *S. epidermidis* (DSM 3269) and an *S. epidermidis* strain isolated from an infected prosthesis were used as test organisms to investigate whether the eluted antibiotics retained antimicrobial activity. The MICs of these strains with standard solutions of the antibiotics are shown in Table S1 in the Supplementary material. Concentration of linezolid and tigecycline eluted at various times from antibiotic-loaded cement samples were determined via LC-MS and the MICs of the eluted antibiotics were determined experimentally (Tables 1 and 2). All eluted tigecycline samples showed activity comparable with the standard solution and established breakpoints[21,22] for all organisms tested (Table 1). The linezolid samples eluted up to 72 h all showed activity comparable to determined MICs and breakpoints against the Gram positive organisms.[21] The linezolid samples eluted over 1 week (168 h) showed higher MICs compared to the other samples and the Gram negative *E. coli* was not inhibited by any of the linezolid samples, as expected (Table 2).

Cytotoxicity of antibiotics towards MG63 cells

The cytotoxic effects of standard solutions of linezolid and tigecycline against MG63 cells were determined using the MTT assay. The addition of increasing concentrations of tigecycline resulted in a marked reduction in cell activity with an IC_{50} between 5 – 10 mg/L. The addition of linezolid showed a small reduction in activity that was not statistically

245 significant ($P > 0.05$). Up to 300 mg/L of linezolid resulted in < 50% reduction in cell activity
246 and so an IC_{50} for linezolid could not be determined (Supplementary material Fig S1).
247 Comparing these results to the concentrations achieved in the elution experiments (Figures 1-
248 4), it is possible that cellular toxicity of tigecycline may be an issue if the *in vivo* eluted
249 concentrations are comparable to those in this laboratory system, whereas linezolid did not
250 show toxicity to mammalian cells, even at substantially higher concentrations than those
251 achieved in the elution experiments.

252 **Impact testing to assess physical strength of bone cements samples**

253 A Charpy type impact test machine was used to evaluate the impact strength of the antibiotic
254 loaded bone cement. Separate bone cement samples loaded either with tigecycline or
255 linezolid at 3 % and 10 % wt/wt were tested, and the results compared to both bone cement
256 without antibiotic and a commercially prepared gentamicin-loaded bone cement, Refobacin®
257 Bone Cement R (Table 3). There was no significant difference in the impact strength of the
258 tigecycline-loaded cement samples at either concentration, compared to the control without
259 antibiotic. The 10% (wt/wt) tigecycline-loaded cement was the only cement that had an
260 impact strength that appeared slightly lower than the bone cement without antibiotic,
261 however that difference was not statistically significant. Further, there was no significant
262 difference between the linezolid-loaded samples at either concentration and the Refobacin®
263 Bone cement R samples ($P > 0.05$). The impact strength of both the 3% and 10% (wt/wt)
264 tigecycline cement samples were significantly less ($P < 0.05$) than, though still comparable
265 to, the commercially available Refobacin® Bone Cement R.

266

267

Discussion

The results presented here indicate that tigecycline and linezolid can be included within bone cement and that the elevated temperatures that occur during the curing stage do not compromise their antimicrobial and biocompatibility properties. Both antibiotics elute to clinically relevant concentrations within the first hour in our laboratory elution system (Fig 1 and 2) and retain antimicrobial activity up to one week later. The concentrations of eluted tigecycline peaked around 1 h (Fig 2) and then declined, presumably due to decomposition of the antibiotic. The MICs for eluted tigecycline based upon the concentrations measured by LC-MS showed results comparable with those determined using standard antibiotic solutions (Table 1; Supplementary material Table S1). The MICs of eluted linezolid, the concentration of which increased progressively throughout the experiment (Fig 1), were comparable with those determined using standard antibiotic solutions over the first 72 h. After 1 week, eluted linezolid showed approximately 5-20-fold higher MICs than the standard linezolid (Table 2; Supplementary material Table S1) , which may indicate slow decomposition of the eluted antibiotic that was not revealed by LC-MS. Previously, Anagnostakos *et al.* reported elution of 1% of total linezolid from bone cement, compared to 3% for gentamicin loaded cement over 8 days and Jackson *et al.* reported up to 3% elution over a 4 week period.[14,15] Cement containing linezolid and gentamicin has shown inhibited growth of methicillin-resistant *S.aureus* for up to 8 days.[14] However as this previous study is in conjunction with gentamicin it does not necessarily confirm the activity of the linezolid on its own.

The effect of wear on the tigecycline-loaded bone cement samples significantly reduces the elution of tigecycline. After 1 hour there was > 4-fold reduction in the elution rate from the worn sample, compared to the unworn control (Fig 3). Conversely, wear has very little effect

293 on the elution of linezolid from the bone with similar elution rates and profiles for both worn
294 and unworn samples (Fig 4). This may be relevant in the clinical application of these systems
295 where the cement surface experiences wear. Previously we have reported similarly
296 contrasting results with gentamicin and daptomycin–loaded bone cements where elution of
297 gentamicin was significantly reduced by wear, yet elution of daptomycin was not affected.[16]
298 In this study it was suggested that crystal size and distribution were the two main factors
299 influencing this difference in elution characteristics between the two antibiotics. It was
300 observed that the larger crystals of gentamicin within the orthopaedic cement created voids
301 on the surface upon contact with the aqueous solution, thus allowing greater deformation of
302 the bone cement surface due to wear. It was further proposed that this deformation prevented
303 the solution from penetrating deep into the bone cement, thereby limiting the amount of
304 antibiotic that can be eluted. In the current study we have shown that the crystals of
305 tigecycline are smaller than the linezolid crystals and so crystal size appears not to be the
306 main factor determining the reduced elution from worn bone cement samples here
307 (Supplementary material Fig S1). However there is a much greater tendency for the
308 tigecycline crystals to aggregate within the cement compared to the linezolid. The surface of
309 the tigecycline loaded cement showed areas of aggregated tigecycline crystals, which may
310 also produce voids upon contact with the aqueous solution and so increase the deformation of
311 the bone cement surface (Supplementary material Fig S2, S3).
312
313 The impact strength of the linezolid and tigecycline loaded cements produced results
314 comparable to those commercially available bone cements. The lowest impact strength was
315 seen in the 10% tigecycline containing cement suggesting that tigecycline may have some
316 effect on the mechanical strength of the cement. A previous study by Kries et al showed the
317 addition of tigecycline had a detrimental effect on compressive and bending strength of

tigecycline-loaded bone cement.[23] Kries et al. also mentioned a 3.8-fold increase in curing time compared to cement only. Curing time was not specifically investigated during the current study, but all cement samples were fully cured within < 1 h.

The MTT assay showed that linezolid had low cytotoxicity towards MG63 cells. Up to 300 mg/L linezolid concentration resulted in <50% loss of cell activity and so an IC_{50} was not determined. Tigecycline showed greater cytotoxicity with an IC_{50} of 5 - 10 mg/L. This result is consistent with the findings of Pina et al.,[24] who also found that tigecycline concentrations >10 mg/L severely affected the cell growth of osteoblastic cells.

Conclusions

The antimicrobial activity of linezolid and tigecycline eluted from within bone cement, reaches therapeutically relevant concentrations within the critical perioperative period (based on a typical arthroplasty operation of 1-2 h). Antimicrobial activity is observed up to 1 week later. However, the concentration of tigecycline added to cement may need to be controlled due to the possible cytotoxicity of the eluted antibiotic towards osteoblast cells. The effect of wear in reducing elution of tigecycline in the laboratory reported here is also a factor to be borne in mind if this antibiotic is used in revision surgery. Owing to ongoing antibiotic resistance problems, there is a need to use antibiotics such as linezolid and tigecycline both alone and in conjunction with other antibiotics (such as gentamicin which is included in commercial bone cement preparations currently widely used in arthroplasty surgery). The current study is an *in vitro* assessment of the performance and do not model the conditions *in vivo*. Upon implantation the prosthetic comes into contact with extracellular fluid, bone and muscle tissue, all of which will affect elution and the local accumulation of antibiotic. Further

work assessing the *in vivo* performance of these cements as well as more mechanical testing needs to be carried out to fully evaluate these antibiotic loaded cements. However, based on the results presented above we propose that linezolid and tigecycline are encouraging candidates for local delivery via antibiotic loaded bone cement, in the treatment and prevention of prosthetic joint infection.

Acknowledgements

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Transparency Declarations

None.

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Figures and tables

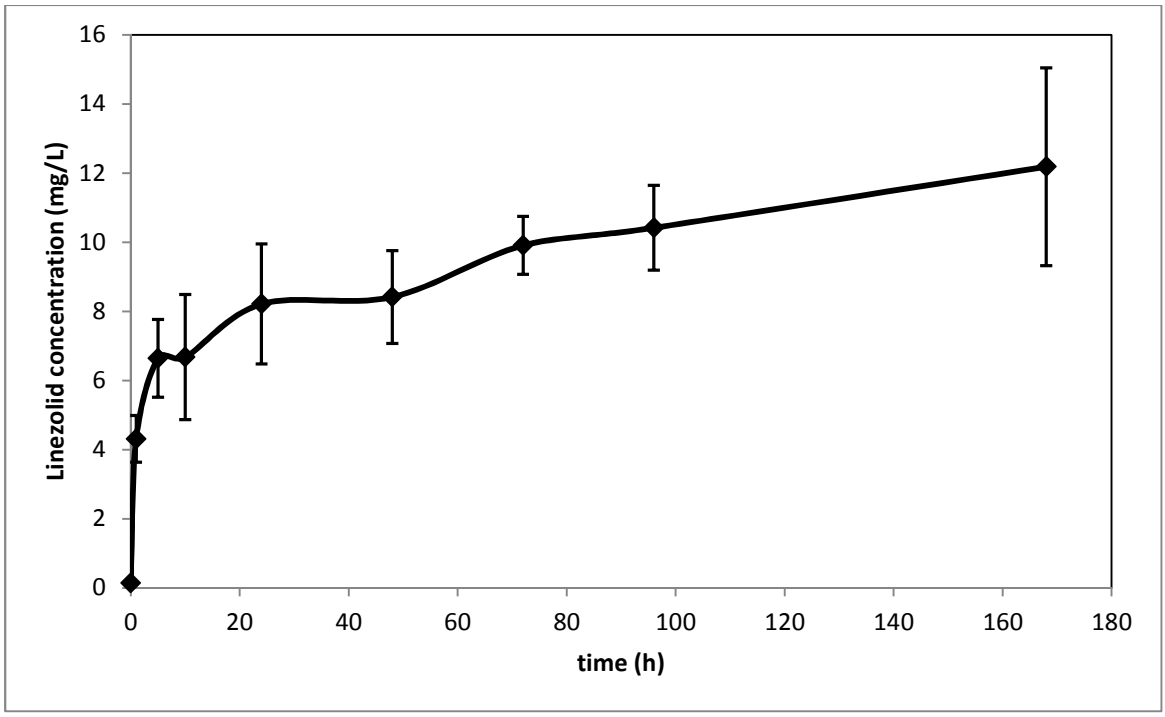


Fig 1: Concentration of linezolid eluted from bone cement over a 1-week period. Results are shown as the mean of three separate experiments ± standard deviation and have been normalised to 1 g bone cement in 5 mL of buffer.

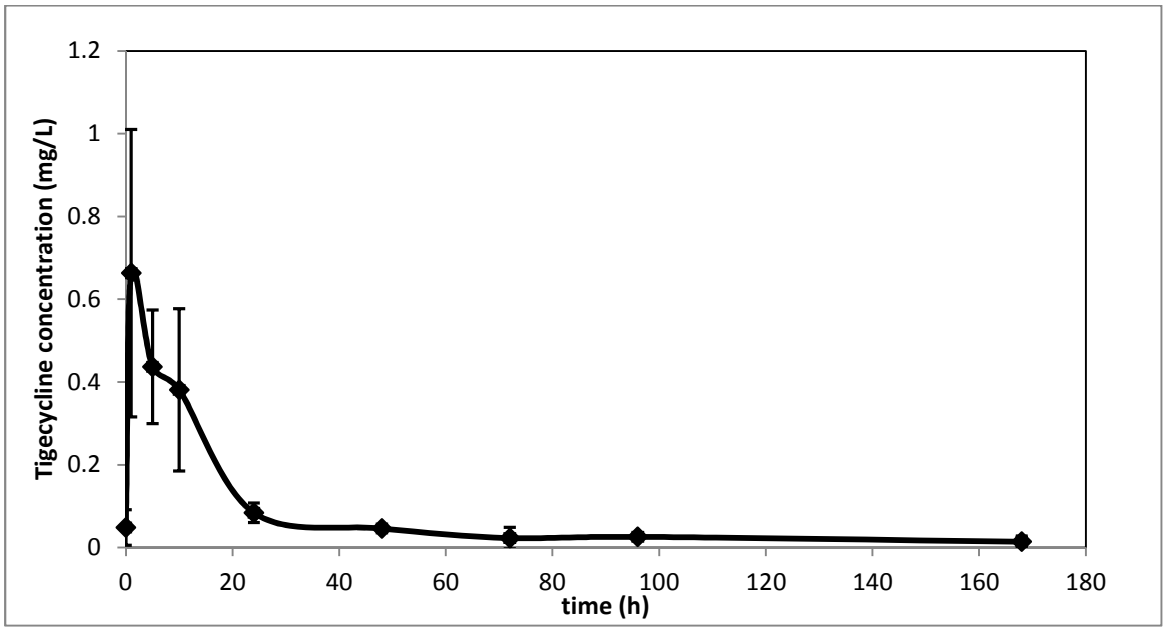
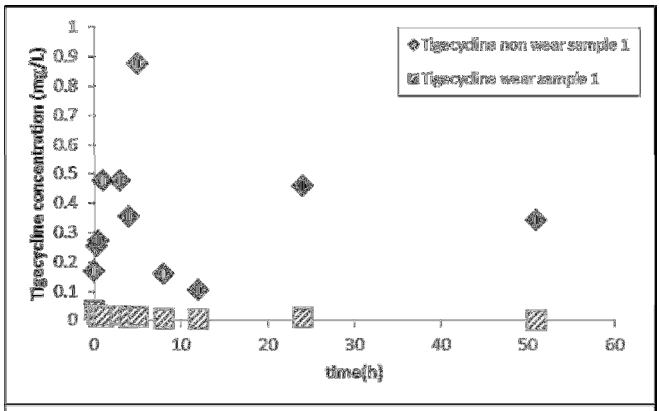


Fig 2: Concentration of tigecycline eluted from bone cement over a 1 week period. Results are shown as the mean of three separate experiments ± standard deviation and have been normalised to 1 g bone cement in 5 mL of buffer.

426

A)



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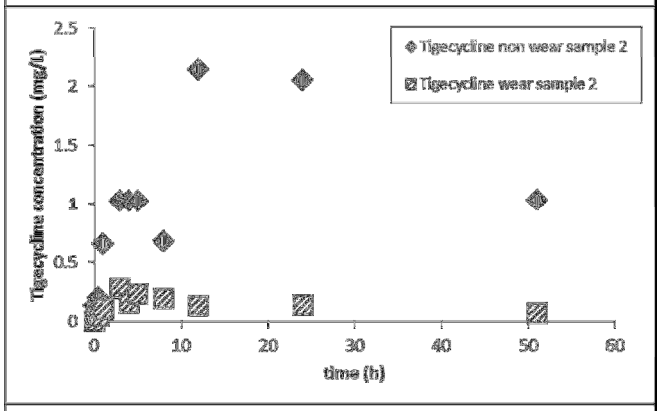

C)

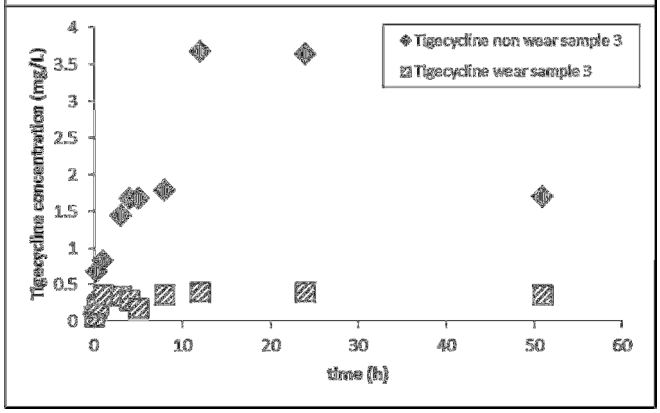

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Fig 3) Results from three separate experiments (A, B and C) comparing elution of tigecycline

429 from worn and unworn tigecycline-loaded bone cement. Concentration of antibiotic was quantified by LCMS.

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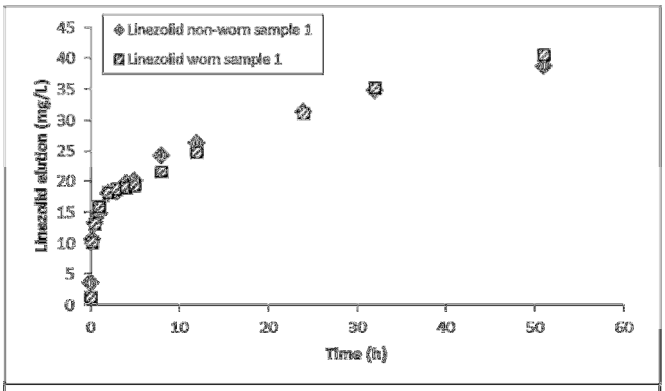
A)



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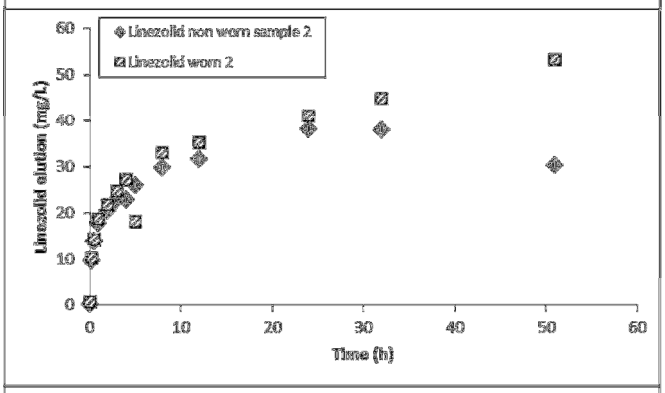

C)

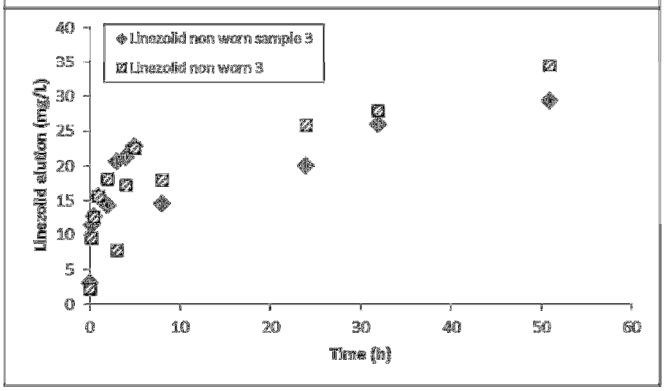

Fig 4) Results from three separate experiments (A, B and C) comparing elution of linezolid from worn and unworn linezolid-loaded bone cement. Concentration of antibiotic was quantified by LCMS.

Organism	1 h eluate (mg/L)	24 h eluate (mg/L)	48 h eluate (mg/L)	72 h eluate (mg/L)	168 h eluate (mg/L)
S.aureus SH1000	0.2	0.1	0.059	0.088	0.044
Methicillin-resistant *S.aureus (clinical isolate)*	<0.10	0.056	0.059	0.088	0.044
S.epidermidis (clinical isolate)	0.41	0.225	0.12	0.18	>0.18
S.epidermidis (DSM 3269)	0.41	0.28	0.12	0.088	0.052
E.coli (clinical isolate)	0.41	0.7	0.24	0.35	>0.18

Table 1:MICs of tigecycline eluted from bone cement, determined by the broth microdilution method. Experiments were carried out in triplicate.

Organism	1h eluate (mg/L)	24 h eluate (mg/L)	48 h eluate (mg/L)	72 h eluate (mg/L)	168 h eluate (mg/L)
S.aureus SH1000	1.9	0.89	0.93	1.06	9.75
Methicillin-resistant *S.aureus (clinical isolate)*	1.9	0.89	0.93	1.06	9.75
S.epidermidis (clinical isolate)	0.95	0.89	0.93/1.88	0.53	9.75
S.epidermidis (DSM 3269)	0.95	0.89	0.93	0.53	9.75
E.coli (clinical isolate)	>15.27	>28.50	>30.00	>34.00	>9.75

Table 2: MICs of linezolid eluted from bone cement, determined by the broth microdilution method.

Experiments were carried out in triplicate..

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Bone cement	1. Impact strength ($kJ.m^2$)
Cement only	0.259 ± 0.0444
3% tigecycline	0.2649 ± 0.0299
10% tigecycline	0.2271 ± 0.0217
3% linezolid	0.3175 ± 0.0422
10% linezolid	0.3187 ± 0.0493
3% gentamicin	0.3205 ± 0.05
10% gentamicin	0.3673 ± 0.0133
Refobacin® Bone Cement R (1.25 % gentamicin)	0.3343 ± 0.0212

456 Table 3: Impact strength of antibiotic loaded bone cements determined using a Charpy-type testing apparatus.

457 Results are shown as a mean of five separate experiments ± standard deviation. Biomet Bone Cement® was used

458 for all preparations unless stated otherwise.

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