Effect of Polysorbate 80 on Oritavancin Binding to Plastic Surfaces: Implications for Susceptibility Testing[▽]

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Oritavancin, a semisynthetic lipoglycopeptide with activity against gram-positive bacteria, has multiple mechanisms of action, including the inhibition of cell wall synthesis and the perturbation of the membrane potential. Approved guidelines for broth microdilution MIC assays with dalbavancin, another lipoglycopeptide, require inclusion of 0.002% polysorbate 80. To investigate the potential impact of polysorbate 80 on oritavancin susceptibility assays, we quantified the recovery of [¹⁴C]oritavancin from susceptibility assay plates with and without polysorbate 80 and examined the effect of the presence of polysorbate 80 on the oritavancin MICs for 301 clinical isolates from the genera *Staphylococcus*, *Enterococcus*, and *Streptococcus*. In the absence of polysorbate 80, [¹⁴C]oritavancin was rapidly lost from solution in susceptibility assay test plates: 9% of the input drug was recovered in broth at 1 h when [¹⁴C]oritavancin was tested at 1 µg/ml. Furthermore, proportionately greater losses were observed at lower oritavancin concentrations, suggesting saturable binding of oritavancin to surfaces. The inclusion of 0.002% polysorbate 80 or 2% lysed horse blood permitted the recovery of 80 to 100% [¹⁴C]oritavancin at 24 h for all drug concentrations tested. Concordantly, oritavancin MIC₉₀s for streptococcal isolates, as determined in medium containing 2% lysed horse blood, were identical with and without polysorbate 80. In stark contrast, polysorbate 80 reduced the oritavancin MIC₉₀s by 16- to 32-fold for clinical isolates of enterococci and staphylococci, which are typically cultured without blood. The results presented here provide evidence that the MIC data for oritavancin in the current literature significantly underestimate the potency of oritavancin in vitro. Moreover, the combination of data from MIC and [¹⁴C]oritavancin recovery studies supports the revision of the oritavancin broth microdilution method to include polysorbate 80 throughout the assay.

Clinical laboratories typically use commercially prepared antibacterial drug dilution panels to generate in vitro susceptibility data. In the past, it was not common knowledge that these commercial products frequently included a wetting agent (e.g., pluronic F or polysorbate [Tween] 80) in the drug diluent to facilitate organism dispersal (17). It was generally believed that although the use of these wetting agents was not strictly in compliance with the methods recommended by the Clinical and Laboratory Standards Institute (CLSI), their use had no appreciable effect on the in vitro activities of various antimicrobial drugs.

The development of dalbavancin, a lipoglycopeptide, initiated a shift in broth dilution susceptibility testing procedures early in the new millennium. A report by members of the dalbavancin Quality Control (QC) Working Group (1) proposed dalbavancin MIC QC ranges for the most commonly used gram-positive reference strains. It was demonstrated that the use of polysorbate 80 was essential for accurate dalbavancin susceptibility testing data, such as that required in clinical microbiology laboratories and for regulatory approval.

The proposed dalbavancin QC guidelines were accepted by the CLSI and appear in the recent revisions of CLSI documents M7-A7 (5) and M100-S18 (6). The broth microdilution

methodology with polysorbate 80 has been used in a number of recent studies with dalbavancin (7, 9, 10, 16). Polysorbate 80 has also been shown to have an effect on the broth microdilution MICs of polymyxins against gram-negative bacteria, reducing the MICs by 2 to 3 doubling dilutions (17). Although polysorbate 80 is widely used in the preparation of commercial broth microdilution drug panels or as part of the inoculum in broth microdilution assays to maintain the solubility of certain antibacterial agents or to ensure their quantitative recovery from solution, its effect on MIC assay results has been systematically evaluated for very few drugs (11).

Oritavancin is a semisynthetic lipoglycopeptide antimicrobial agent with potent activity against a range of drug-resistant gram-positive bacteria, including vancomycin- and methicillin-resistant staphylococci, vancomycin-resistant enterococci, and penicillin-resistant streptococci (8, 15, 19). The development of newer drugs that show activity against drug-resistant isolates is timely, given the increased incidence of isolation of such isolates in the clinic. Unlike other glycopeptide antibiotics, such as vancomycin and teicoplanin, oritavancin exhibits in vitro antibacterial activity that is rapidly bactericidal and concentration dependent (13, 14, 20).

The current M7-A7 guidelines of the CLSI recommend oritavancin preparation and broth microdilution assay in the absence of polysorbate 80 (5). While a recent revision to the M100 document (document M100-S18) recommends the inclusion of polysorbate 80 in susceptibility tests for oritavancin (6), the oritavancin MIC data in the literature have been generated without the use of polysorbate 80. Prompted by the

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FIG. 1. [^{14}C]oritavancin recovery in CAMHB with or without supplements from 96-well plates versus time ([^{14}C]oritavancin at $1\ \mu\text{g}/\text{ml}$). P80, 0.002% polysorbate 80.

available information on the use of polysorbate 80 in dalbavancin susceptibility testing, we investigated the influence of polysorbate 80 on the results of oritavancin susceptibility assays. In this study, we performed broth microdilution oritavancin MIC assays with and without polysorbate 80 with both reference and clinical isolates. Comparator antibiotics were used in parallel to establish the specificity of the effect of polysorbate 80. These studies were extended to determine the recovery of radiolabeled oritavancin with and without polysorbate 80 or 2% lysed horse blood (LHB) in susceptibility assay test plates in an attempt to elucidate the observed specific reductions in oritavancin MICs with polysorbate 80.

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MATERIALS AND METHODS

Bacterial strains. Strains were isolated from clinical specimens obtained from patients. The *in vitro* activities of oritavancin, vancomycin, and teicoplanin were tested against 301 gram-positive clinical isolates of enterococci ($n = 100$), staphylococci ($n = 102$), and streptococci ($n = 99$) collected from the United States and Europe. Approximately 50% of each of the species selected were from the Eurofins surveillance repository (Eurofins Medinet, Anti-Infective Services, Herndon, VA), and the other 50% were selected from a collection of isolates used in a clinical trial of oritavancin.

Drugs. Ciprofloxacin hydrochloride was purchased from MP Biomedicals (Solon, OH). Teicoplanin was purchased from Sequoia Research Products Limited (Pangbourne, United Kingdom). Vancomycin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Oritavancin diphosphate was from Targanta Therapeutics (Cambridge, MA).

Recovery of oritavancin from microtiter plates. To quantitate the recovery of oritavancin from microtiter plates under conditions simulating broth microdilution assays, [^{14}C]oritavancin diphosphate (specific activity, 53 mCi/mmol) and [^{14}C]ciprofloxacin (specific activity, 20 mCi/mmol) (Moravak Biochemicals and Radiochemicals, Brea, CA) were used. The radiolabeled drugs were dissolved in water or in 0.002% polysorbate 80 in water, diluted in cation-adjusted Mueller-Hinton broth (CAMHB) or CAMHB plus 2% LHB in the presence and absence of 0.002% polysorbate 80, and dispensed into 96-well polystyrene plates (Sensititre, product number S-4100; TREK Diagnostic Systems) without cells. Recovery of the radiolabeled agents was assessed by scintillation counting (MicroBeta TriLux 1450; Perkin-Elmer) of the supernatant over time to yield the residual counts relative to the counts of the input label at time zero.

Direct assessment of oritavancin binding to microtiter plate surfaces. Binding of [^{14}C]oritavancin and [^{14}C]ciprofloxacin to surfaces was directly assessed in a solid-phase radioligand binding (scintillation proximity) assay with a Flashplate microtiter plate (catalog number SMP200; Perkin-Elmer Life and Analytical Sciences, Waltham, MA). The specific activities of [^{14}C]oritavancin and [^{14}C]ciprofloxacin were adjusted with unlabeled drug to normalize the input counts for each drug at each test concentration; scintillation counting of an aliquot of each drug verified the normalization. The drugs were diluted in CAMHB over a range

FIG. 2. Oritavancin recovery in CAMHB with or without polysorbate 80 in 96-well plates versus oritavancin concentration. P80, 0.002% polysorbate 80.

of concentrations (2 to $16\ \mu\text{g}/\text{mL}$), and $100\ \mu\text{l}$ of each dilution was dispensed into the wells of a Flashplate microtiter plate. The plate was incubated for 16 h, covered, at ambient temperature. The number of counts per well emanating from the proximity of the radiolabel to the surface-bound scintillant in the Flashplate microtiter plate was obtained by counting in the MicroBeta TriLux scintillation counter without the use of liquid scintillation cocktail. Samples were then removed, the wells were washed four times with $300\ \mu\text{l}$ of CAMHB, and the residual scintillation proximity signal was determined to assess whether the initial counts originated from drug binding to the well surfaces or from drug precipitation.

MIC determination. MICs were tested by broth microdilution according to the CLSI M7-A7 methodology (5). The procedure was modified to include polysorbate 80 (final test concentration, 0.002%) in some assays. When the drugs were tested in polysorbate 80, the drugs were dissolved in 0.002% polysorbate 80 in water, and polysorbate 80 was maintained at this concentration in drug dilution steps as well as in the MIC assays. The following reference strains were used: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Streptococcus pneumoniae* ATCC 49619.

Titration of polysorbate 80 in oritavancin MIC test. To determine the polysorbate 80 concentration dependence of the oritavancin MICs, broth microdilution susceptibility tests with *S. aureus* ATCC 29213 as an indicator strain were performed according to the CLSI M7-A7 methodology (5), except that various test concentrations of polysorbate 80 were included at the drug dissolution step and were maintained at the test concentration onwards.

Order of addition of polysorbate 80 in oritavancin MIC test. To determine whether the order of addition of polysorbate 80 affected the oritavancin MICs, broth microdilution susceptibility tests were performed with *S. aureus* ATCC 29213 as an indicator strain, in which oritavancin was either dissolved in 0.002% polysorbate 80 and diluted and assayed by maintaining polysorbate 80 at 0.002% or dissolved and diluted in water and then assayed by adding inoculum with or without polysorbate 80. When polysorbate 80 was present, it was added at a final concentration of 0.002%, as described in the CLSI guidelines for dalbavancin (5, 6).

RESULTS

Polysorbate 80 and LHB promote recovery of oritavancin. Scintillation counting and determination of oritavancin recovery in CAMHB from polystyrene microtiter plates revealed a rapid and profound loss of oritavancin ($9\% \pm 1\%$ recovery at 1 h) and nearly complete recovery ($88\% \pm 8\%$ at 24 h) in the presence of 0.002% polysorbate 80 (Fig. 1). Possible explanations for the loss of oritavancin in the absence of polysorbate 80 include oritavancin binding to the surfaces of the test vessel and oritavancin precipitation from solution. To address the idea of limiting solubility, the recovery of [^{14}C]oritavancin at 2 h was determined over a range of drug concentrations. Oritavancin recovery in the absence of polysorbate 80 was proportionately lowest when low concentrations of oritavancin were tested (Fig. 2). This finding argues against limiting solu-

FIG. 3. Solid-phase radiolabel binding (scintillation proximity) assay in Flashplate microtiter plate to assess binding of [¹⁴C]oritavancin and [¹⁴C]ciprofloxacin to surfaces. Equivalent counts of [¹⁴C]oritavancin and [¹⁴C]ciprofloxacin were used for each specific drug concentration. [¹⁴C]Ori, [¹⁴C]oritavancin; [¹⁴C]Cip, [¹⁴C]ciprofloxacin.

bility, since such an idea predicts that the greatest proportional losses of oritavancin to precipitation would be expected at higher concentrations of drug. In contrast to the results for oritavancin, [¹⁴C]ciprofloxacin was quantitatively recovered, regardless of the presence or absence of polysorbate 80 (97% ± 4% recovery at 24 h with [¹⁴C]ciprofloxacin at 1 µg/ml in the absence of polysorbate 80; data not shown). In sum, the findings suggest a rapid and profound binding of oritavancin to the surface of the assay plate.

To further distinguish between explanations of the limiting solubility of oritavancin and the binding of oritavancin to the surfaces of the test vessels, the effect of adding LHB, a component of the CLSI-recommended medium for the growth of streptococci (6), was tested. LHB was found to promote the nearly quantitative recovery of oritavancin in CAMHB from the wells of the microtiter plate (Fig. 1). While the means by which LHB promotes oritavancin recovery is not completely understood, it may be similar to that of polysorbate 80, putatively through the blocking of nonspecific oritavancin binding sites on the surfaces of the test vessels.

Binding to Flashplate microtiter plate. A solid-phase (scintillation proximity) assay was used to assess directly whether radiolabeled oritavancin bound to microtiter plate surfaces. Figure 3 shows the scintillation proximity signals emanating from [¹⁴C]oritavancin and [¹⁴C]ciprofloxacin in the 96-well Flashplate microtiter plate before and after washes, as assessed

by counting without added liquid scintillation cocktail. The [¹⁴C]oritavancin counts were concentration dependent, reaching 2,847 ± 258 cpm at 16 µg/ml drug. Furthermore, a significant proportion (64%) of the [¹⁴C]oritavancin scintillation proximity signal was maintained after the washing step. In contrast, the counts did not exceed 245 ± 12 cpm for 16 µg/ml [¹⁴C]ciprofloxacin before the washing step and were reduced to background levels (~0 cpm) after the washing step. These observations suggest that [¹⁴C]oritavancin, but not [¹⁴C]ciprofloxacin, bound directly to the surface of the Flashplate microtiter plate.

Broth microdilution MICs against reference strains in the presence and absence of 0.002% polysorbate 80 and 2% LHB.

The broth microdilution MICs for oritavancin and the comparator drugs were determined for the susceptibility testing reference strains by using CLSI-recommended media: CAMHB for *S. aureus* and *E. faecalis* and CAMHB containing 2 to 5% LHB for *S. pneumoniae* (5, 6). The MICs of the glycopeptides vancomycin and teicoplanin and of the fluoroquinolone ciprofloxacin for *S. aureus* ATCC 29213 were unaffected by polysorbate 80 (Table 1). However, a striking shift in the oritavancin MIC for this strain was noted with polysorbate 80, for which the MIC decreased 32-fold in its presence (Table 1). Similarly, the oritavancin MIC for *E. faecalis* ATCC 29212 decreased 16-fold in the presence of 0.002% polysorbate 80 compared to the MIC when 0.002% polysorbate 80 was absent, whereas the vancomycin, teicoplanin, and ciprofloxacin MICs for this strain were unaffected by polysorbate 80. In contrast, against *S. pneumoniae* ATCC 49619, the oritavancin MIC in CLSI-recommended medium (CAMHB containing 2 to 5% LHB) was unaffected (within a doubling dilution) by polysorbate 80. This finding suggests that either a strain- or a medium-specific effect could account for the observed lack of impact of polysorbate 80 on the oritavancin MIC for this strain.

To further investigate whether 2% LHB had an effect similar to that of polysorbate 80 in reducing oritavancin MICs, oritavancin MICs were determined against the pneumococcal reference strain by using brain heart infusion broth. Although this medium is not recommended by the CLSI for susceptibility tests with streptococci, its ability to readily support *S. pneumoniae* growth in the absence of 2% LHB allowed the direct testing of the effect of polysorbate 80 on oritavancin MICs. The

TABLE 1. MICs for oritavancin and comparators against reference strains in different media in the presence and absence of polysorbate 80 or LHB

ATCC reference strain (medium)	MIC (µg/ml) for antibacterial agents with and without polysorbate 80 ^a							
	Oritavancin		Vancomycin		Teicoplanin		Ciprofloxacin	
	–P80	+P80	–P80	+P80	–P80	+P80	–P80	+P80
<i>S. aureus</i> ATCC 29213 (CAMHB)	1	0.03	1	1	0.5	0.5	0.5	0.5
<i>S. aureus</i> ATCC 29213 (CAMHB + 2% LHB)	0.03	0.03	1	1	1	0.5	0.5	0.5
<i>E. faecalis</i> ATCC 29212 (CAMHB)	0.5	0.03	4	4	0.5	0.5	0.5	0.5
<i>E. faecalis</i> ATCC 29212 (CAMHB + 2% LHB)	0.016	0.03	2	4	1	0.5	0.5	0.5
<i>S. pneumoniae</i> ATCC 49619 (CAMHB + 2% LHB)	0.001	0.002	0.25	0.25	0.03	0.03	0.5	1
<i>S. pneumoniae</i> ATCC 49619 (BHI)	0.008	0.001	0.25	0.25	0.03	0.03	0.5	0.5
<i>S. pneumoniae</i> ATCC 49619 (BHI + 2%LHB)	0.001	0.001	0.25	0.25	0.03	0.03	0.5	1

^a MICs obtained by using CLSI-recommended conditions, as described in documents M7-A7 (5) and M100-S18 (6), are in boldface and fall within the recommended QC ranges for each drug-QC organism combination (6). CLSI guidelines for oritavancin broth microdilution tests require testing in the presence of 0.002% polysorbate 80. CLSI guidelines for vancomycin, teicoplanin, and ciprofloxacin susceptibility tests do not include polysorbate 80. +P80, with 0.002% polysorbate 80; –P80, without polysorbate 80.

FIG. 4. Titration of polysorbate 80 in oritavancin MIC test with *S. aureus* ATCC 29213.

oritavancin MICs for *S. pneumoniae* ATCC 49619 in brain heart infusion broth were reduced fourfold in the presence of polysorbate 80 or 2% LHB (Table 1). This result argues against the idea that *S. pneumoniae* is in some manner “resistant” to the effect of polysorbate 80 with respect to oritavancin MICs. Interestingly, the inclusion of 2% LHB in place of 0.002% polysorbate 80 in oritavancin susceptibility tests with the *S. aureus* and *E. faecalis* QC strains resulted in 32-fold reductions in the oritavancin MICs for both strains (Table 1). Together, these findings support the assertion that LHB has an effect similar to that of polysorbate 80 in reducing oritavancin MICs.

Titration of polysorbate 80 in broth microdilution assays. To determine the minimum concentration of polysorbate 80 required to mediate the shift in oritavancin MICs, a range of polysorbate 80 concentrations was tested in broth microdilution assays against *S. aureus* ATCC 29213. Figure 4 shows that concentrations of polysorbate 80 above 0.002% provide no further reduction in the oritavancin MIC. However, at 0.001% polysorbate 80, a 1-dilution increase in the MIC was observed; further reduction of the polysorbate 80 concentration below 0.001% yielded progressively elevated MICs (Fig. 4).

Effect of order of addition of polysorbate 80 on oritavancin susceptibility test results. To determine at what step in the broth microdilution methodology the addition of polysorbate 80 maximally reduces the loss of oritavancin to plastic surfaces, we examined the effect that the addition of 0.002% polysorbate 80 either at the last step (in the inoculum, as recommended for dalbavancin [5, 6, 16]) or at the first step (in the solvent used to dissolve oritavancin diphosphate powder and thereafter maintained at 0.002%) had on the oritavancin MIC for *S. aureus* ATCC 29213. The results were compared to the oritavancin MIC obtained in the absence of polysorbate 80. While addition of polysorbate 80 to the inoculum did reduce the MIC 4-fold relative to the MIC under no-polysorbate 80 condition, the greatest reduction in the oritavancin MIC (16-fold) was achieved when polysorbate 80 was present in the solvent and was maintained at 0.002% throughout the testing (Fig. 5).

Broth microdilution MICs of clinical isolates with and without polysorbate 80. The presence of 0.002% polysorbate 80 from the drug dissolution step onwards in broth microdilution assays significantly reduced the oritavancin MICs and MIC₉₀s for enterococci and staphylococci (Table 2). The oritavancin MIC₉₀ was 32 times lower for *S. aureus* and 16 times lower for

FIG. 5. Order of addition of polysorbate 80 (P-80; 0.002%, when present) determines the oritavancin MIC for *S. aureus* ATCC 29213.

coagulase-negative staphylococci when they were tested in the presence of 0.002% polysorbate 80 than when they were tested in the absence of 0.002% polysorbate 80 (Table 2). Likewise, the oritavancin MIC₉₀ was 16 times lower for both *E. faecalis* and *E. faecium* when they were tested in the presence of 0.002% polysorbate 80 than when they were tested in the absence of 0.002% polysorbate 80 (Table 2). The broth microdilution MICs for vancomycin and teicoplanin, in contrast, were generally unaffected (i.e., the MIC ranges and MIC₉₀s were unchanged or were within 1 doubling dilution) by the addition of 0.002% polysorbate 80 for the staphylococcal, enterococcal, and streptococcal isolates tested (Table 2). The only exception was with the teicoplanin MIC₉₀ for *E. faecalis* in the presence of polysorbate 80, in which a modest fourfold decrease in the MIC₉₀ compared to that in the absence of polysorbate 80 was observed. While the basis for this is not yet clear, it is important to note that this observation for teicoplanin was specific for *E. faecalis* clinical isolates and was not observed for staphylococci or the *E. faecium* clinical isolates (Table 2) or for *E. faecalis* reference strain ATCC 29212 (Table 1). On the contrary, shifts in the oritavancin MIC₉₀s in the presence of 0.002% polysorbate 80 were observed for all staphylococcal and enterococcal isolates in this study. The oritavancin broth microdilution MICs were consistently lower than the MICs for vancomycin or teicoplanin when they were tested against staphylococci and enterococci, provided that oritavancin MICs were measured in the presence of 0.002% polysorbate 80 (Table 2). For streptococci, the oritavancin MIC₉₀s (Table 2) did not change in the presence of 0.002% polysorbate 80 compared with those obtained without 0.002% polysorbate 80.

Overall, the impact of polysorbate 80 on the oritavancin MICs for the clinical isolates was consistent with the results of both the broth microdilution assays with the ATCC reference strains and recovery studies with [¹⁴C]oritavancin. The 16- to 32-fold reductions in the oritavancin MICs for the staphylococci and enterococci in the presence of polysorbate 80 are likely a result of polysorbate 80 inhibition of the binding of oritavancin to plastic surfaces; the absence of a shift in the oritavancin MIC against streptococci in the presence of polysorbate 80 is likely linked to the presence of LHB, which affords protection against oritavancin losses to the surfaces of the test vessels.

TABLE 2. In vitro activities of oritavancin and the comparator drugs against clinical isolates of enterococci, staphylococci, and streptococci by broth microdilution with and without polysorbate 80

Organism	Agent(s)	MIC ($\mu\text{g/ml}$)		MIC ₉₀ /P80 ^c MIC ₉₀ ratio
		Range	90%	
<i>E. faecalis</i> (<i>n</i> = 70)	Oritavancin	0.12–4	2	16
	Oritavancin-P80	0.008–0.5	0.12	
	Teicoplanin	0.06–64	0.5	4
	Teicoplanin-P80	0.06–64	0.12	
	Vancomycin	0.12–>256	2	1
	Vancomycin-P80	0.5–>256	2	
<i>E. faecium</i> (<i>n</i> = 30)	Oritavancin	0.12–4	4	16
	Oritavancin-P80	0.004–0.5	0.25	
	Teicoplanin	0.12–256	64	0.5
	Teicoplanin-P80	0.06–128	128	
	Vancomycin	0.25–>256	>256	1
	Vancomycin-P80	0.25–>256	>256	
<i>S. aureus</i> (<i>n</i> = 76)	Oritavancin	1–8	4	32
	Oritavancin-P80	0.015–0.25	0.12	
	Teicoplanin	0.12–1	0.5	1
	Teicoplanin-P80	0.12–2	0.5	
	Vancomycin	0.5–1	1	1
	Vancomycin-P80	0.5–2	1	
CoNS ^a (<i>n</i> = 26)	Oritavancin	0.12–8	4	16
	Oritavancin-P80	0.008–0.5	0.25	
	Teicoplanin	0.12–4	2	1
	Teicoplanin-P80	0.06–4	2	
	Vancomycin	0.5–2	1	1
	Vancomycin-P80	0.5–2	2	
<i>S. agalactiae</i> ^b (<i>n</i> = 29)	Oritavancin	0.03–0.5	1	
	Oritavancin-P80	0.03–0.5	0.25	
	Teicoplanin	0.03–0.12	0.06	1
	Teicoplanin-P80	0.03–0.12	0.06	
	Vancomycin	0.25–0.5	0.5	1
	Vancomycin-P80	0.25–0.5	0.5	
<i>S. pneumoniae</i> (<i>n</i> = 19)	Oritavancin	0.00025–0.004	0.004	1
	Oritavancin-P80	0.0005–0.004	0.004	
	Teicoplanin	0.15–0.06	0.06	1
	Teicoplanin-P80	0.15–0.06	0.06	
	Vancomycin	0.12–0.5	0.25	1
	Vancomycin-P80	0.12–0.5	0.25	
<i>S. pyogenes</i> (<i>n</i> = 29)	Oritavancin	0.015–0.5	0.25	1
	Oritavancin-P80	0.015–0.5	0.25	
	Teicoplanin	0.001–0.25	0.06	2
	Teicoplanin-P80	0.015–0.06	0.03	
	Vancomycin	0.25–0.5	0.25	1
	Vancomycin-P80	0.25–0.5	0.25	
Viridans group streptococci (<i>n</i> = 14)	Oritavancin	0.004–2	1	1
	Oritavancin-P80	0.004–2	1	
	Teicoplanin	0.002–0.12	0.12	0.5
	Teicoplanin-P80	0.002–0.25	0.12	
	Vancomycin	0.25–1	0.5	1
	Vancomycin-P80	0.25–0.5	0.5	
Group C and G streptococci (<i>n</i> = 8)	Oritavancin	0.004–1	NA	NA ^d
	Oritavancin-P80	0.004–1	NA	
	Teicoplanin	0.03–0.06	NA	NA
	Teicoplanin-P80	0.03–0.06	NA	
	Vancomycin	0.25–0.5	NA	NA
	Vancomycin-P80	0.12–0.5	NA	

^a CoNS, coagulase-negative staphylococci.^b Streptococcal MICs were tested in the presence of 2% LHB.^c P80, 0.002% polysorbate 80.^d NA, not applicable, i.e., the MIC₉₀ was not calculated for groups containing <10 isolates.

DISCUSSION

The use of radiolabeled oritavancin in MIC assay plates allowed the quantitative assessment of oritavancin recovery from solution as a function of time. Oritavancin recovery from solution decreased rapidly in the absence of polysorbate 80 or LHB and was promoted with their inclusion. The apparent correlation between the oritavancin concentration and the proportional recovery of the drug without polysorbate 80 suggests saturable binding. The idea that polysorbate 80 may act by enhancing or maintaining oritavancin solubility is also countered with these results, since one would predict greater losses to insolubility at higher, and not lower, concentrations of the drug. This finding supports the notion that polysorbate 80 exerts its effect principally by reducing the adsorptive loss of oritavancin rather than by augmenting oritavancin solubility.

Also noteworthy is that the effect of polysorbate 80 in protecting oritavancin from adsorptive losses was independent of the concentration of oritavancin, at least over the range of 1 to 16 $\mu\text{g/ml}$. Extension of these ideas to consider the potential impact of oritavancin surface binding characteristics in the absence of polysorbate 80 predicts that very small proportional losses would be expected at high concentrations of oritavancin, such as those found following the reconstitution of the drug product for clinical administration (10 mg/ml).

That oritavancin binds to plastic surfaces was demonstrated directly in a scintillation proximity assay in which the binding of [^{14}C]oritavancin directly to the surface of the Flashplate microtiter plate yielded a significant scintillation proximity signal without scintillation cocktail. The high residual counts that were retained after a wash step to remove precipitated or loosely associated material further served to confirm the idea that oritavancin binds to microtiter plate surfaces rather than precipitates during the course of the assay. Together, these results concur with the results of recovery assays, in which ciprofloxacin but not oritavancin was quantitatively recovered from Sensititre microtiter plates in the absence of polysorbate 80.

The loss of drug to plastic surfaces in susceptibility tests has similarly been reported for ramoplanin, a glycolipodepsipeptide complex with potent *in vitro* activity against gram-positive bacteria (18). The loss of ramoplanin to plastic surfaces was minimized by including 0.02% bovine serum albumin in the tests (4, 12, 18).

The effect of polysorbate 80 on oritavancin MICs was studied in an effort to determine whether the observed rapid, saturable binding of oritavancin to test vessel surfaces had a functional consequence. The 32- and 16-fold reductions in oritavancin broth microdilution MICs against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212, respectively, observed in the presence of polysorbate 80 are consistent with the differential recovery of [^{14}C]oritavancin from 96-well microtiter plates in the presence and the absence of polysorbate 80.

Polysorbate 80 must be present at 0.002% throughout all steps of the broth microdilution assay, from drug dissolution, dilutions, and the final assay, to maximize oritavancin recovery and therefore to best represent its potency. This represents an important methodological difference compared to the methodology for dalbavancin, for which MICs are identical whether polysorbate 80 is added in the drug broth dilutions or in the

inoculum water (16). The results from the order-of-addition experiment also support the idea that polysorbate 80 reduces oritavancin binding to plastic surfaces rather than synergistically acts mechanistically with the drug: in the case of synergistic activity, the order of addition of polysorbate 80 would not have been expected to affect the oritavancin MICs, given that the final concentration of polysorbate 80 was identical whether it was added in the inoculum or in the solvent and maintained thereafter.

The oritavancin MIC for *S. pneumoniae* ATCC 49619 was unaffected (within a doubling dilution) by 0.002% polysorbate 80. This finding may be rationalized by results from the [^{14}C]oritavancin experiments, in which LHB, a component of the CLSI-recommended pneumococcal growth medium, was found to substitute for polysorbate 80 in promoting the recovery of radiolabeled drug from solution. The CLSI now recommends the inclusion of 0.002% polysorbate 80 throughout oritavancin drug dissolution, dilution, and susceptibility testing of staphylococci, enterococci, and streptococci (6). This should limit the variability of the MICs for all three genera, since time- and concentration-dependent variations in oritavancin recovery during drug preparation will be predictably minimized.

LHB (2%) was added to CAMHB in place of polysorbate 80 in susceptibility tests with *S. aureus* and *E. faecalis* reference strains. The results from that experiment clearly indicated that 2% LHB had an effect similar to that of polysorbate 80 in promoting the recovery of oritavancin from solution.

The effect of 0.002% polysorbate 80 was specific to oritavancin, since the broth microdilution MICs of the comparator glycopeptides vancomycin and teicoplanin in the presence of 0.002% polysorbate 80 were generally unchanged or within a doubling dilution of their MIC in its absence. This finding again argues against the idea that polysorbate 80 nonspecifically increases the potencies of glycopeptides and lipoglycopeptides. A similar conclusion was made in a study evaluating the effect of 0.002% polysorbate 80 on the *in vitro* activity of tigecycline (11): the MICs of tigecycline, vancomycin, and teicoplanin were identical or within 1 doubling dilution in the presence and the absence of 0.002% polysorbate 80.

The results presented here demonstrate that polysorbate 80 addition is necessary during broth microdilution testing to most accurately represent the antibacterial activity of oritavancin, since in its absence, oritavancin concentrations are drastically lower than those intended for almost the entire duration of the 24-h broth microdilution assay. An extension of this idea is that oritavancin susceptibility assays that were performed by the broth microdilution methodology without polysorbate 80, prior to quantitative determinations of [^{14}C]oritavancin binding to labware surfaces, likely significantly underestimated the potency of oritavancin. Our studies strongly support the inclusion of 0.002% polysorbate 80 during dissolution of the oritavancin drug powder and in all subsequent steps of the broth microdilution method. Furthermore, the results from our recovery studies indicate that investigators should exercise significant caution in *in vitro* and *in vivo* experiments with oritavancin to ensure proper assessment of the intended concentrations of the drug in their systems.

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