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Analysis of MIC and Disk Diffusion Testing Variables for Gepotidacin and Comparator Agents against Select Bacterial Pathogens

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ABSTRACT This study was conducted to determine the effect of testing parameters on the *in vitro* activity of gepotidacin, a new triazaacenaphthylene antibacterial agent for the treatment of conventional and biothreat pathogens. CLSI methods, and variations of those methods, were used to test 10 *Staphylococcus aureus*, 10 *Streptococcus pneumoniae*, 10 *Haemophilus influenzae*, and 5 *Escherichia coli* isolates by MIC and 30 *S. aureus*, 15 *S. pneumoniae*, and 15 *S. pyogenes* isolates by disk diffusion (DD) methods. Levofloxacin and linezolid were tested as comparator agents for MIC and DD methods, respectively. Broth microdilution (BMD), macrodilution (MD), and agar dilution (AD) methods were compared. Variations in media, temperature, incubation time, CO_2 level, and inoculum concentration were tested by all methods, and variations in pH, calcium, magnesium, zinc, potassium, thymidine, and polysorbate 80 levels were tested by BMD and DD. The addition of albumin, serum, and lung surfactant was studied by BMD. The variables that impacted the results the most were high inoculum and pH 5.5 (no growth of *H. influenzae* and *S. pneumoniae* by BMD). Gepotidacin AD MIC levels were increased and disk zone diameters were decreased for all species in 10% CO_2 incubation. The following variables had a minimal effect on gepotidacin results: pH, agar method, atmospheric condition, temperature, and addition of serum and albumin for broth methods. There were also some slight differences in gepotidacin disk results between disk manufacturers and some agar types and also with potassium and thymidine for *S. pneumoniae*. For all other variations, gepotidacin MIC and disk results were considered comparable to reference results.

KEYWORDS MIC, antimicrobial susceptibility, disk diffusion, gepotidacin, testing variables

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Gepotidacin (GSK2140944), first in the novel triazaacenaphthylene class of bacterial type IIA topoisomerase inhibitors, inhibits bacterial DNA replication by a novel mechanism and has *in vitro* activity against susceptible and drug-resistant pathogens associated with a range of conventional and biothreat infections.

In performing antimicrobial susceptibility testing, it is important to control various testing conditions. Prior *in vitro* studies for numerous antimicrobial agents have shown the effect of various factors on the susceptibility test result (1–14). Standardized methods, such as the MIC and disk diffusion method established by the Clinical and Laboratory Standards Institute (CLSI), were developed in order to define important test variables and optimal testing conditions (15–17). This study was undertaken to determine the reproducibility of the reference methods when testing the same organism over several days and also to determine the effect of various testing parameters on the *in vitro* activity of gepotidacin and a comparative agent by MIC against *Staphylococcus*

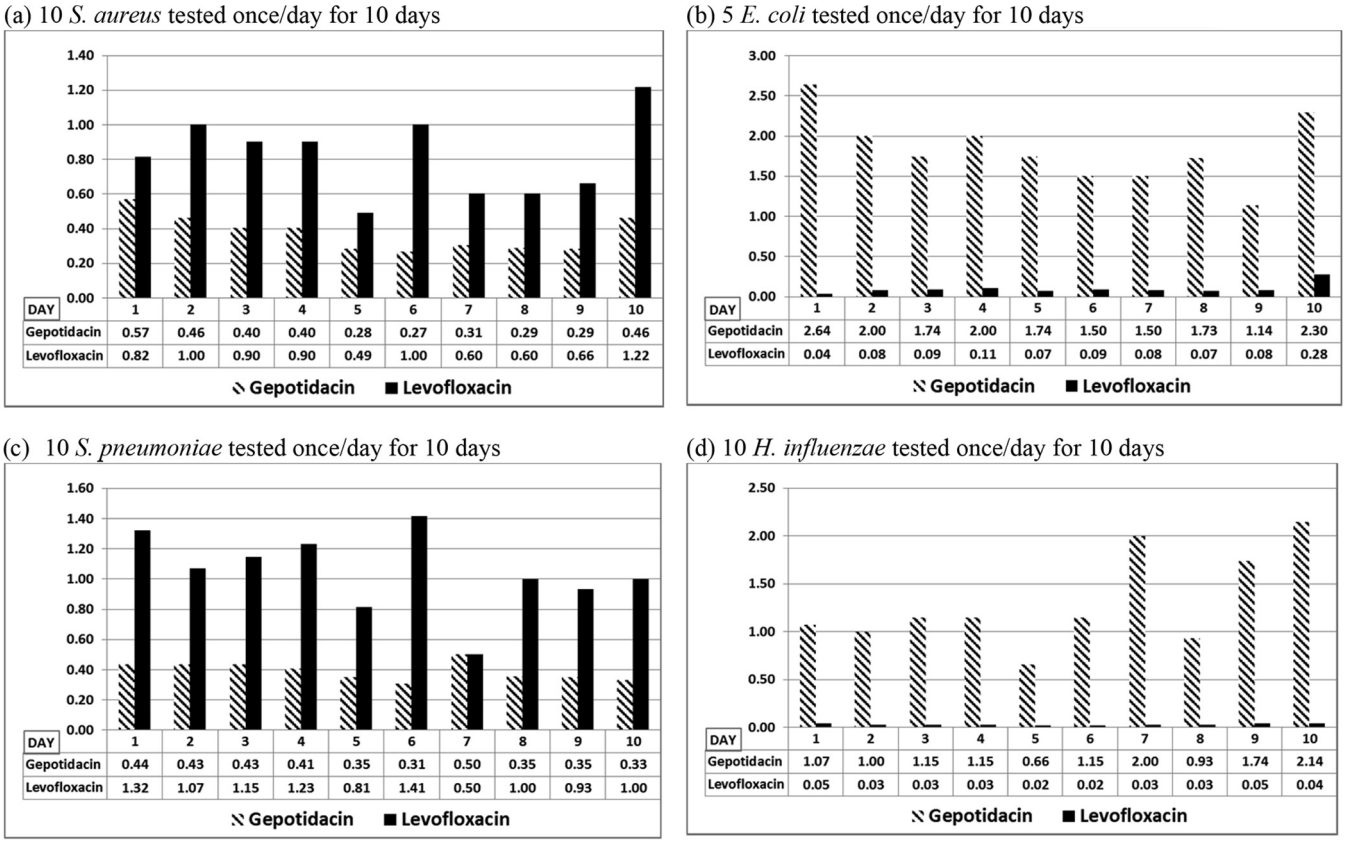


FIG 1 Reproducibility of CLSI broth microdilution method (geometric mean MICs [μg/ml] of gepotidacin and levofloxacin for multiple isolates tested on multiple days during the study period).

aureus, *Escherichia coli*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* and by disk diffusion against *S. aureus*, *S. pneumoniae*, and *Streptococcus pyogenes*.

RESULTS

MIC. For each MIC method and method variable tested, growth was satisfactory according to CLSI M07, unless otherwise indicated. Gepotidacin mean MIC results by the reference broth microdilution (BMD) method varied by approximately 1 dilution over 10 days, with the exception of *H. influenzae* mean MIC results, which varied by approximately 2 dilutions (Fig. 1). Gepotidacin BMD MIC results for the four quality control (QC) strains were within CLSI established ranges, with the exception of 1 out-of-range result each for *H. influenzae* ATCC 49247 (gepotidacin MIC of 2 μg/ml) and *S. pneumoniae* ATCC 49619 (gepotidacin MIC of 0.5 μg/ml). Levofloxacin mean MIC results varied by approximately 1 dilution for *S. aureus*, *S. pneumoniae*, and *H. influenzae* and by approximately 2 dilutions for *E. coli*. All levofloxacin BMD MIC results for the four quality control strains were within CLSI established ranges. While the majority of gepotidacin macrodilution (MD) and agar dilution (AD) MIC results were within 1 doubling dilution of the BMD MIC results for all study isolates, *S. aureus* gepotidacin AD MIC results were approximately 1.2 doubling dilutions higher than BMD MIC results (Table 1).

Mean dilution differences and essential agreement percentages for those method variations that were shown to have essential agreement percentages of $<$90% are shown in Tables 1 and 2. Although incubation temperatures of 30°C and 40°C did not impact MIC results for most species tested, gepotidacin MIC results were lower for *S. pneumoniae* and *H. influenzae* incubated at 40°C than for the reference BMD at 35°C (mean dilution differences, -1.0 and -2.5, respectively). There was no difference in BMD MIC results attributed to atmospheric conditions; however, higher gepotidacin AD

TABLE 1 *In vitro* activity of gepotidacin and levofloxacin: comparison of the CLSI MIC broth microdilution reference method to variations in testing conditions

Parameter or condition	Comparative variable	Mean dilution difference[a] (% essential agreement[b])							
		Gepotidacin				Levofloxacin			
		S. aureus ($n = 10$)	*S. pneumoniae* ($n = 10$)	*H. influenzae* ($n = 10$)	*E. coli* ($n = 5$)	*S. aureus* ($n = 7$[c])	*S. pneumoniae* ($n = 10$)	*H. influenzae* ($n = 10$)	*E. coli* ($n = 5$)
MIC method	MD	√	√	√	√	√	√	√	0.4[d] (80.0)
	AD	1.2 (60.0)	√	√	√	√	√	√	√
Medium[e]	ISB	√	NA	NA	√	√	NA	NA	√
	BSB	NA	√	NA	NA	NA	√	NA	NA
	BSB + NAD	NA	NA	√	NA	NA	NA	√	NA
	EUB	NA	NA	√	NA	NA	NA	√	NA
Temperature (°C)	30	√	√	√	√	−1.2 (71.4)	√	−0.4 (80.0)	√
	40	√	−1.0 (80.0)	−2.5[d] (30.0)	√	√	√	√	√
Incubation time (h)	16	√	√	√	√	√	√	√	√
	24[e]	√	√	√	√	√	√	√	√
	48	√	√	√	√	√	√	√	√
Inoculum (CFU/ml)	10^4	√	√	√	√	√	√	√	√
	10^6	√	√	√	√	√	√	√	√
	10^7	6.9[d] (0.0)	6.6[d] (0.0)	3.9[d] (0.0)	4.8[d] (0.0)	1.9[d] (85.7)	6.0[d] (0.0)	6.7[d] (0.0)	1.6[d] (40.0)
Atmosphere (% CO_2)	5	√	√	√	√	√	√	√	√
	10	√	√	√	√	√	√	√	√
Calcium (μg/ml)	50	√	√	√	√	√	√	√	√
	100	√	√	√	√	√	√	√	√
Magnesium (μg/ml)	+25	√	√	√	√	√	1.4 (60.0)	√	√
	+50	√	√	√	√	1.5 (42.9)	√	√	√
Potassium (mmol/liter)	+12.5	√	√	√	√	−0.1 (85.7)	√	√	√
	+25	√	√	√	√	√	√	√	√
	+50	√	√	√	√	√	√	√	√
Zinc (mmol/liter)	+2	√	√	√	√	√	√	√	√
	+5	√	√	√	√	0.6 (85.7)	√	√	√
	+10	√	√	√	√	−0.2 (85.7)	√	√	√
Thymidine (μg/ml)	+1	√	√	√	√	√	√		√
	+5	√	√	√	√	√	√	√	√
pH	5.5	1.4 (40.0)	NG	NG	1.4 (60.0)	√	NG	NG	√
	6.5	√	1.8 (20.0)	√	√	√	√	√	√
	8.5	√	−1.6 (50.0)	−2.6 (0.0)	−1.2 (40.0)	√	√	√	√
% serum	25	−0.7 (80.0)	√	√	√	√	√	√	√
	50	−0.5 (80.0)	−1.6 (50.0)	√	−1.0 (80.0)	0.6 (85.7)	√	√	√
Albumin (μg/dl)	+4	1.0 (60.0)	√	√	0.4 (80.0)	√	√	√	√
% lung surfactant	1	√	√	√	0.6 (80.0)	√	√	√	√
	5	√	√	√	√	0.01 (85.7)	√	√	√
% polysorbate 80	0.002	√	√	√	√	√	√	√	√

[a]Dilution differences were calculated for each MIC by subtracting the $\log_2 + 10$ test MIC from the $\log_2 + 10$ reference MIC, and mean dilution differences were determined for each method. MD, macrodilution; AD, agar dilution; ISB, IsoSensitest broth; BSB, ISB–5% LHB; BSB-NAD, ISB–5% LHB–20 mg/liter NAD; EUB, CAMHB–5% LHB–20 mg/liter NAD (NAD). √, essential agreement ≥ 90%; NG, no growth; NA, not applicable.

[b]Data represent percentages of MICs by the reference and comparative methods within ±1 doubling dilution.

[c]Some strains not included because reference MICs were off the scale.

[d]Value might have been greater than reported due to the presence of an isolate(s) with an off-scale MIC value(s).

[e]For *S. pneumoniae* and *H. influenzae*, the reference condition was 24 h of incubation time (h) and the tested variable was 20 h.

MIC results were obtained for *S. aureus* incubated in 5% CO_2 and in 10% CO_2 than were obtained with the reference AD under ambient conditions (mean dilution differences, 1.6 and 1.7, respectively). Gepotidacin AD MIC results were also higher for *S. pneumoniae* incubated in 10% CO_2 (mean dilution difference, 0.7) and for *E. coli* incubated in 5% CO_2 and 10% CO_2 (mean dilution difference, 0.8) than those obtained under the

TABLE 2 *In vitro* activity of gepotidacin and levofloxacin: comparison of the CLSI MIC agar dilution reference method to variations in testing conditions

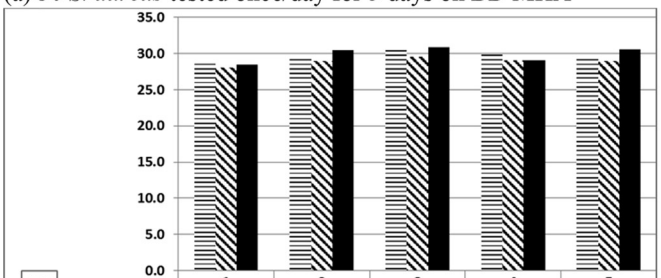
Parameter or condition	Comparative variable	Mean dilution difference[a] (% essential agreement[b])					
		Gepotidacin			Levofloxacin		
		S. aureus ($n = 10$)	*S. pneumoniae* ($n = 10$)	*E. coli* ($n = 5$)	*S. aureus* ($n = 7$[c])	*S. pneumoniae* ($n = 10$)	*E. coli* ($n = 4$[c])
Medium[e]	ISA	0.7 (80.0)	NA	√	√	NA	−0.4 (80.0)[d]
	BSA	NA	√	NA	NA	√	NA
	EUA	NA	√	NA	NA	√	NA
Temperature (°C)	30	√	√	√	√	√	√
	40	√	√	√	√	√	√
Incubation time (h)	16	√	√	√	√	√	√
	24[e]	√	√	√	√	√	√
	48	√	√	√	√	√	√
Inoculum (CFU/spot)	2×10^3	√	√	√	√	√	√
	2×10^5	√	√	√	√	√	√
	2×10^6	5.8[d] (0.0)	2.8[d] (0.0)	3.4[d] (0.0)	5.1[d] (0.0)	3.1[d] (0.0)	8.4[d] (20.0)
Atmosphere (% CO_2)	5[e]	1.6 (30.0)	√	0.8 (80.0)	1.3 (66.7)	√	√
	10	1.7 (40.0)	0.7 (80.0)	0.8 (80.0)	1.7 (66.7)	√	√

ISA, IsoSensitest agar; BSA, MHA–5% defribrinated horse blood; EUA, MHA–5% defribrinated horse blood–20 mg/liter NAD.
[a]Dilution differences were calculated for each MIC by subtracting the $\log_2 + 10$ test MIC from the $\log_2 + 10$ reference MIC, and mean dilution differences were determined for each method.
[b]Percentage of MICs by the reference and comparative methods within ± 1 doubling dilution.
[c]Some strains not included because reference MICs were off the scale.
[d]Value may be greater than reported due to the presence of an isolate(s) with an off-scale MIC value(s).
[e]For *S. pneumoniae* 24 h and 5% CO_2 incubation is the reference condition and 20 h and ambient is the tested variable.
√ = Essential agreement ≥ 90%; NA, not applicable.

reference AD conditions for each organism. MIC results were especially impacted as a result of the use of various inoculum concentrations. For all species tested (with the exception of *H. influenzae* and agar dilution, a combination which was not tested), the use of an inoculum concentration of 10^7 CFU/ml for BMD and 2×10^6 CFU/spot for AD resulted in gepotidacin and levofloxacin BMD MIC results that were significantly higher than the MIC results for the reference BMD and AD methods (the majority of MIC results were off the scale at above the highest dilution tested and were indicative of a >16-fold increase in MIC). Changes in pH also had an impact on gepotidacin BMD MIC results. For *S. aureus* and *E. coli*, a pH of 5.5 resulted in increased gepotidacin MIC results compared to reference MIC results (mean dilution difference, 1.4). For *S. pneumoniae* and *H. influenzae*, a pH of 5.5 resulted in no growth in the gepotidacin- or levofloxacin-containing wells or in the positive-growth control well. At a pH of 6.5, *S. pneumoniae* gepotidacin MIC results were higher than reference MIC results (mean dilution difference, 1.8). At a pH of 8.5, *E. coli*, *S. pneumoniae*, and *H. influenzae* gepotidacin MIC results were lower than the reference MIC results (mean dilution differences, −1.2, −1.6, and −2.6, respectively). The addition of 25% serum decreased gepotidacin MIC results for *S. aureus* (mean dilution difference, −0.7), and the addition of 50% serum also decreased gepotidacin MIC results for *E. coli*, *S. pneumoniae*, and *S. aureus* compared to reference MIC results (mean dilution differences, −1.0, −1.6, and −0.5, respectively). The addition of 4 μg/dl of albumin increased gepotidacin MICs compared to reference MIC results (increase of 1 mean dilution).

Disk diffusion. For each disk method and method variable tested, growth was satisfactory according to CLSI M02, unless otherwise indicated. The reference gepotidacin (10 μg) and linezolid (30 μg) mean disk zone diameters differed over 7 days by 2.8 and 2.4 mm for *S. aureus*, 6.7 and 2.7 mm for *S. pneumoniae*, and 2.9 and 2.3 mm for *S. pyogenes*, respectively (Fig. 2). Mean reference method BD zone diameters for *S. aureus* tested over 5 days and for *S. pneumoniae* tested over 7 days were an average of 0.8 mm larger than MAST (Hardy Diagnostics, Santa Maria, CA) zone diameters, and BD

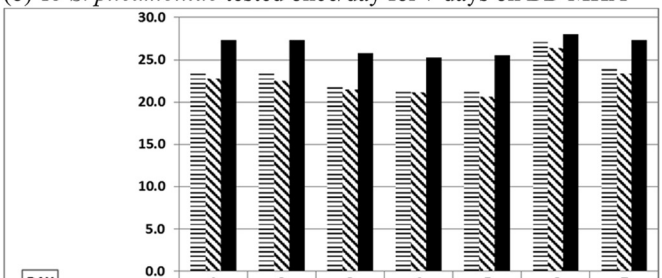
(a) 30 *S. aureus* tested once/day for 5 days on BD MHA



DAY	1	2	3	4	5
Gepotidacin 10 µg BD	28.8	29.5	30.9	29.9	29.7
Gepotidacin 10 µg Mast	28.1	29.0	29.6	29.1	29.0
Linezolid 30 mcg	28.5	30.5	30.9	29.1	30.6

≡ Gepotidacin 10 µg BD ⍀ Gepotidacin 10 µg Mast ■ Linezolid 30 mcg

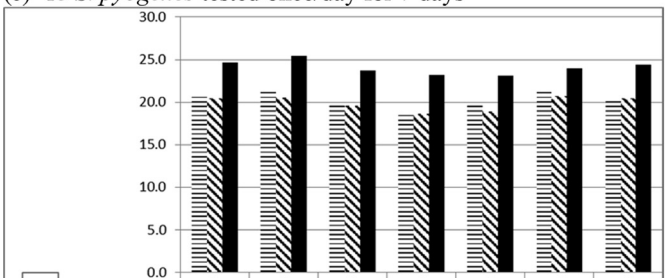
(b) 15 *S. pneumoniae* tested once/day for 7 days on BD MHA



DAY	1	2	3	4	5	6	7
Gepotidacin 10 µg BD	23.5	23.6	22.1	21.5	21.5	27.3	24.2
Gepotidacin 10 µg Mast	22.8	22.5	21.5	21.1	20.6	26.4	23.4
Linezolid 30 mcg	27.3	27.3	25.8	25.3	25.5	28.0	27.3

≡ Gepotidacin 10 µg BD ⍀ Gepotidacin 10 µg Mast ■ Linezolid 30 mcg

(c) 15 *S. pyogenes* tested once/day for 7 days



DAY	1	2	3	4	5	6	7
Gepotidacin 10 µg BD	20.8	21.5	19.9	18.7	19.7	21.4	20.5
Gepotidacin 10 µg Mast	20.4	20.5	19.6	18.6	18.9	20.7	20.4
Linezolid 30 mcg	24.7	25.4	23.7	23.2	23.1	24.0	24.4

≡ Gepotidacin 10 µg BD ⍀ Gepotidacin 10 µg Mast ■ Linezolid 30 mcg

FIG 2 Reproducibility of CLSI disk diffusion method (mean zone diameters [mm] of gepotidacin and linezolid by CLSI disk diffusion for multiple isolates tested on multiple days during the study period).

zone diameters for *S. pyogenes* tested over 7 days were an average of 0.5 mm larger than MAST zone diameters. All gepotidacin 10 μg BD disk results for the two quality control strains were within CLSI established ranges. There were two outlier gepotidacin (10 μg) MAST disk results (21 mm) for *S. pneumoniae* ATCC 49619.

Mean zone diameter differences and essential agreement percentages for those method variations which were shown to have essential agreement percentages of $<$90% are shown in Table 3. Mean zone diameter differences and essential agreement percentages are not shown for method variations that were in agreement with those determined by the reference method (essential agreement, $\geq$ 90%). Among the results from the medium and method study, gepotidacin zone diameters for *S. aureus* determined by the use of a MAST disk on Oxoid Mueller-Hinton agar (MHA) were lower than the reference zone diameters (mean zone difference, $-$2.8 mm). Gepotidacin zone diameters for *S. pyogenes* determined by the use of a MAST disk on MHA–5% defibrinated horse blood (Cleveland Scientific, Bath, OH)–20 mg/liter NAD (EUA) were larger than reference zone diameters (mean zone difference, 1.7 mm). With the exception of *S. aureus* with the MAST disk, gepotidacin zone diameters were larger with ISA using the British Society for Antimicrobial Chemotherapy (BSAC) inoculum for all isolates (mean zone differences: *S. aureus*, 2.1 mm; *S. pneumoniae* = 3.6 and 3.7 mm; *S. pyogenes*, 5.5 and 5.3 mm [for BD and MAST disks, respectively]). A pH of 5.5 reduced gepotidacin zone diameters for *S. aureus* (mean zone differences, $-$4.0 and $-$5.2 mm for BD and MAST disks, respectively). Growth was diminished and gepotidacin zone diameters for streptococci were increased with media at pH 5.5 (mean zone differences: *S. pneumoniae*, 6.1 and 5.4 mm for BD and MAST disks, respectively; *S. pyogenes*, 3.0 for the gepotidacin BD disk). Gepotidacin zone diameters for *S. aureus* on media with a pH of 8.5 were increased compared to reference zone diameters (mean zone differences,

TABLE 3 *In vitro* activity of gepotidacin and linezolid: comparison of the CLSI disk diffusion reference method to variations in testing conditions

Parameter or condition	Comparative variable	Mean zone diam (mm) difference[a] (% essential agreement[b])								
		S. aureus (n = 30)			*S. pneumoniae* (n = 15)			*S. pyogenes* (n = 15)		
		Gepotidacin (10-μg disk)		Linezolid (30-μg disk)	Gepotidacin (10-μg disk)		Linezolid (30-μg disk)	Gepotidacin (10-μg disk)		Linezolid (30-μg disk)
		BD	MAST		BD	MAST		BD	MAST	
Medium	Oxoid MHA[c]	√	−2.8 (83.3)	√	√	√	√	√	√	√
	Hardy MHA[c]	√	√	√	√	√	√	√	√	√
	ISA (CLSI inoculum)[d,e]	0.1 (86.7)	√	√	√	√	√	√	√	√
	ISA (BSAC inoculum)[d,f]	2.1 (83.3)	√	4.5 (56.7)	3.6 (86.7)	3.7 (80.0)	4.4 (66.7)	5.5 (33.3)	5.3 (26.7)	7.1 (6.7)
	EUA	NA	NA	NA	√	√	√	√	1.7 (86.7)	√
Temperature (°C)	30	√	√	√	√	√	4.8 (40.0)	4.2 (66.7)	3.6 (80.0)	3.5 (73.3)
	40	−0.9 (80.0)	−0.9 (80.0)	−4.2 (50.0)	−3.1 (80.0)	−2.8 (80.0)	−2.8 (86.7)	5.2 (53.3)	4.7 (53.3)	√
Incubation time (h)[g]	14	√	√	√	√	√	√	4.4 (60.0)	3.9 (73.3)	3.7 (66.7)
	24	√	√	√	√	√	√	√	√	√
	48	√	√	√	√	√	√	√	√	√
Inoculum (CFU/ml)	10^6	4.5 (66.7)	4.5 (46.7)	5.6 (36.7)	6.6 (6.7)	6.1 (13.3)	6.4 (6.7)	5.5 (33.3)	5.6 (20.0)	6.9 (0.0)
	10^7	3.2 (83.3)	3.0 (83.3)	3.2 (80.0)	3.9 (66.7)	3.5 (66.7)	4.1 (46.7)	3.8 (80.0)	3.8 (73.3)	4.9 (33.3)
	10^9	√	√	−2.6 (80.0)	√	√	−3.0 (86.7)	√	√	√
Atmosphere (% CO_2)	5[h]	√	√	√	−1.1 (80.0)	√	√	√	√	√
	10	−3.6 (63.3)	−3.9 (60.0)	√	−5.6 (33.3)	−5.5 (20.0)	−2.1 (86.7)	−3.4 (86.7)	√	√
Calcium (μg/ml)	50	√	√	√	√	√	√	√	√	√
	100	√	√	√	√	√	√	√	√	√
Magnesium (μg/ml)	+12.5	√	√	√	√	√	√	√	√	√
	+25	√	√	√	√	√	√	√	√	√
Potassium (mmol/liter)	+12.5	√	√	√	−0.4 (86.7)	−0.2 (86.7)	(73.3)	√	√	√
	+25	√	√	√	−0.1 (80.0)	0.0 (80.0)	0.1 (73.3)	√	√	√
	+50	√	√	√	−0.1 (66.7)	0.13 (73.3)	√	√	√	√
Zinc (mmol/liter)	+2	√	√	√	√	√	√	√	√	√
	+5	0.9 (86.7)	√	√	√	√	√	√	√	√
	+10	√	√	√	√	√	2.9 (86.7)	√	√	√
Thymidine (μg/ml)	+1	√	√	√	−3.7 (53.3)	−3.1 (66.7)	√	√	√	√
	+5	√	√	√	−3.6 (73.3)	−3.4 (66.7)	−2.3 (86.7)	√	√	√
pH	5.5	−4.0 (50.0)	−5.2 (30.0)	3.2 (60.0)	6.1 (20.0)	5.4 (33.3)	12.0 (13.3)	√	3.0 (73.3)	2.1 (86.7)
	6.5	√	√	√	√	√	√	√	√	√
	8.5	13.6 (0.0)	12.7 (0.0)	8.2 (30.0)	√	2.3 (80.0)	√	√	√	√
% polysorbate 80	0.002	√	√	√	√	√	√	√	√	√

[a]Mean zone diameters were determined for each comparative variable method and subtracted from the mean zone diameter of the reference results from the same day of testing. MHA, Mueller-Hinton agar; ISA, IsoSensitest agar; EUA, MHA–5% defribrinated horse blood–20 mg/liter NAD. √, essential agreement ≥ 90%; NA, not applicable.

[b]Data represent percentages of zone diameters by the reference method and comparative method within ± 4 mm.

[c]For *S. pneumoniae* and *S. pyogenes*, MHA was supplemented with 5% sheep blood (SBA).

[d]For *S. pneumoniae* and *S. pyogenes*, ISA was supplemented with 5% defibrinated horse blood (BSA).

[e]The CLSI inoculum was a 0.5 McFarland standard.

[f]The BSAC inoculum dilutions used were 1:10 for *S. aureus* and *S. pneumoniae* and 1:100 for beta-hemolytic streptococci.

[g]For *S. pneumoniae* and *S. pyogenes*, the reference condition was 20 to 24 h of incubation time and the comparative variables were 16, 18, and 48 h.

[h]For *S. pneumoniae* and *S. pyogenes*, the reference condition was 5% CO_2 incubation time and the comparative variable was ambient atmosphere.

13.6 and 12.7 mm for BD and MAST disks, respectively), and gepotidacin zone diameters for *S. pyogenes* were also increased (mean zone difference, 2.3 mm). Zinc, added at a concentration of 5 mmol/liter, increased zone diameters for gepotidacin and *S. aureus* (mean zone difference, 0.9 mm for the BD disk). The inoculum had a significant impact on disk diffusion results. Gepotidacin zone diameters were larger at inoculum concentrations of 10^6 CFU/ml for all isolates (mean zone differences ranging from 4.5 to 6.1 mm) and 10^7 CFU/ml (mean zone differences ranging from 3.0 to 3.9 mm). With the exception of the MAST disk for *S. pyogenes*, a reduction in gepotidacin zone diameters

for all isolates occurred when plates were incubated in a 10% CO_2 atmosphere (mean zone differences ranging from −3.4 to −5.6). Gepotidacin zone diameters were also reduced at 5% CO_2 for *S. pneumoniae* by the BD disk method (mean zone difference, −1.1 mm). Gepotidacin zone diameters for *S. pneumoniae* were reduced with addition of 1 μg/ml thymidine (mean zone differences, −3.7 and −3.6 mm for BD and MAST disks, respectively) and 5 μg/ml thymidine (mean zone differences, −3.1 and −3.4 mm for BD and MAST disks, respectively). A reduced incubation time of 14 h resulted in larger gepotidacin zone diameters for *S. pyogenes* (mean zone differences, 4.4 and 3.9 mm for BD and MAST disks, respectively). An incubation temperature of 40°C decreased gepotidacin zone diameters for *S. aureus* and *S. pneumoniae* (mean zone differences ranging from −0.9 to −3.1 mm); however, in contrast, a temperature of 40°C resulted in increased gepotidacin zone diameters for *S. pyogenes* (mean zone differences, 5.2 and 4.7 mm for BD and MAST disks, respectively). When plates were incubated at a reduced temperature of 30°C, gepotidacin zone diameters also increased for *S. pyogenes* (mean zone differences, 4.2 and 3.6 mm for BD and MAST disks, respectively). Agar supplementation with 12.5, 25, and 50 mmol/liter of potassium decreased gepotidacin zone diameters for *S. pneumoniae* (mean zone differences ranging from 0 to −0.4 mm), with the exception of the addition of 50 mmol/liter potassium for the MAST disk, which increased the mean gepotidacin zone diameter by 0.13 mm.

Result summary. When the study isolates were tested according to the reference methods on multiple days during the course of the study, average gepotidacin MIC results for all isolates were within an approximately 2-dilution range and average gepotidacin disk results (with the exception of *S. pneumoniae* results) were within an approximately 2-mm range. Greater variations in gepotidacin reference disk diffusion results were observed for *S. pneumoniae* (average, 5.8-mm range). For all species tested, a high inoculum level was shown to impact gepotidacin and comparator MIC and disk diffusion results to the greatest degree. A variation of the media pH was also shown to affect results; pH 5 affected the growth of streptococcus and *H. influenzae* in general, as well as *S. aureus* and *E. coli* results; pH 8.5 affected gepotidacin results for all species, with the exception of *S. aureus* MIC and *S. pyogenes* disk results. Incubation in 10% CO_2 for agar dilution and disk diffusion affected gepotidacin results for all species. Other factors that had a minimal effect on gepotidacin results were agar dilution for *S. aureus*, incubation at 40°C for all species, with the exception of *S. aureus* and *E. coli* by BMD, and the addition of serum to BMD for *S. aureus* and *S. pneumoniae* and albumin for *S. aureus*. In addition, disk results differed slightly between disk manufacturers (larger zones for BD disks), on the various media (smaller zones on Oxoid MHA, larger on EUA media, and larger on ISA with BSAC inoculum), and with addition of potassium and thymidine for *S. pneumoniae*. For the majority of the study isolates, all other variables tested had no significant effect on gepotidacin MIC or disk results.

DISCUSSION

Reference susceptibility testing methods are standardized; for some drugs, however, this biological assay can be significantly impacted by slight changes in testing conditions. While the standardized method includes accepted ±1-dilution variability, drug- and organism-specific studies are necessary in order to understand this variation. This is especially important when testing and reporting results from clinical isolates and determining susceptibility interpretive criteria (18). In this study, the impact of testing variables on gepotidacin susceptibility was evaluated. For most organisms, there was good agreement (within ±1 dilution or ±4 mm) between results on multiple days when gepotidacin and comparators were tested by the reference standardized broth microdilution, agar dilution, and disk diffusion methods. Any minor MIC or zone differences attributed to day-to-day variation were addressed by performing the reference method on the same day as the variable testing method.

Polysorbate 80 is used as a surfactant for testing of some antibacterial agents and may also be used in commercial antimicrobial susceptibility systems at a concentration of 0.002 μg/ml; therefore, it is important to understand any impact on the *in vitro*

activity of new antimicrobial agents when polysorbate 80 is utilized. Gepotidacin and levofloxacin BMD MICs and gepotidacin and linezolid zone diameters were not impacted by the addition of 0.002 μg/ml polysorbate 80 in this study. Another important variable to consider is the effect of lung surfactant on the MIC. The effect of lung surfactant should be studied for any agent that is being developed for respiratory indications, as the impact of this variable was initially studied and determined to be an issue for the use of daptomycin (19). Gepotidacin and levofloxacin MIC results were not affected by the addition of 1% and 5% of the lung surfactant utilized in this study.

With exception of the 16-h read time variable for the *S. aureus* and *E. coli* BMD method, the levels of each variable tested in this study were in contrast to those recommended by and utilized in standard published method guidelines, such as those from CLSI and EUCAST (15–17) (http://www.eucast.org/ast_of_bacteria/). Therefore, if the standard method is followed, the reproducibility of gepotidacin MIC and disk results should be similar to what was observed in this study for the reference methods. For most laboratories, reproducibility is assessed with QC organisms and surveillance of MIC and zone diameter distributions for clinical isolates. If QC results are out of range or trending, the factors that may be impacting the susceptibility results should be assessed. For gepotidacin, the inoculum is an important factor to consider, although a substantial (2 $\log_{10}$) increase in the inoculum concentration would be required for a significant MIC change. Since gepotidacin agar dilution MIC results for *S. aureus* were approximately 1.5 dilutions higher than the broth microdilution results, this should be taken into consideration in evaluating agar dilution results, especially if MIC results are near a susceptible/resistant breakpoint.

As gepotidacin is tested and used clinically, it is important to understand the impact of the various factors in performing susceptibility tests. The results of this study further emphasize the importance of adhering to standardized testing methods in conducting susceptibility testing for antimicrobials, including gepotidacin.

MATERIALS AND METHODS

The reference MIC methods (BMD, AD, and MD) as well as the different media were initially tested for both MIC and disk methods. CLSI BMD and disk diffusion methods were then modified according to specific method variables. The variables tested were those that could potentially affect gepotidacin susceptibility results as experienced with other antimicrobial agents. Lung surfactant was added to determine if there was any impact on the *in vitro* activity of gepotidacin in consideration of potential respiratory indications. The MIC study was performed initially, and the disk diffusion study followed; therefore, not all isolates or variables tested were similar. The bacterial species chosen for testing were those targeted for potential indications for gepotidacin at the time that the studies were conducted. The studies were performed over several days, and the reference method was tested once on each day of testing along with the method variations studied that day for each isolate. In addition to the assessment of the impact of the variable modifications, this allowed assessment of the reproducibility of the reference broth microdilution (BMD) and disk diffusion (DD) methods over time. The reference method and variation of method procedures by organism species are summarized in Table 4 and 5.

MIC. Initially, 10 *S. aureus* isolates (5 methicillin-susceptible and 5 methicillin-resistant strains, including 1 glycopeptide-intermediate and 1 glycopeptide-resistant strain), 5 *E. coli* isolates (including 1 cefixime-resistant, 1 extended β-lactamase-producing, and 1 carbapenemase-producing strain), 10 *S. pneumoniae* isolates (3 penicillin-susceptible, 3 penicillin-intermediate, and 4 penicillin-resistant strains, including 2 multidrug-resistant strains), and 10 *H. influenzae* isolates (5 β-lactamase-negative strains, including 1 ampicillin-resistant strain, and 5 β-lactamase-positive strains, including 1 tetracycline-resistant strain) were tested using 3 different MIC methods (BMD, MD, and AD) for gepotidacin and levofloxacin (15, 16). The effects of the choice of media and other method variables were then determined by broth microdilution and agar dilution methods. Quality control strains (*S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. pneumoniae* ATCC 49619, and *H. influenzae* ATCC 49247) were tested each day for all reference and variable MIC methods, and the results were compared to CLSI expected ranges (16).

Broth microdilution. The effect of media was determined for the BMD method by using cation-adjusted Mueller-Hinton broth (CAMHB; Becton Dickinson, Sparks, MD) and IsoSensitest broth (ISB; Oxoid, Basingstoke, United Kingdom) against *S. aureus* and *E. coli*; CAMHB–5% lysed horse blood (LHB; Cleveland Scientific, Bath, OH) and ISB–5% lysed horse blood (BSB) against *S. pneumoniae*; and *Haemophilus* Test Medium (HTM; Remel, Lenexa, KS), CAMHB–5% lysed horse blood–20 mg/liter NAD (EUB; Sigma-Aldrich, St. Louis, MO), and ISB–5% lysed horse blood–20 mg/liter NAD (BSB-NAD) against *H. influenzae*. The following significant variables were studied for all study isolates by BMD: temperature, incubation time, atmospheric conditions, and inoculum concentration, pH, calcium, magnesium, zinc, potassium, thymidine, polysorbate 80, albumin, serum, and lung surfactant levels. All medium additives were filter

TABLE 4 Reference method and variation of MIC methods studied

Parameter or condition	Broth microdilution (BMD) result					Agar dilution (AD) result				
	Test organism(s)	Reference method	Variables studied			Test organism(s)	Reference method	Variables studied		
			1	2	3			1	2	3
MIC method	All	BMD	MD	AD						
Medium	*S. aureus*, *E. coli*	CAMHB	ISB			*S. aureus*, *E. coli*	MHA	ISA		
	S. pneumoniae	LHB	BSB			*S. pneumoniae*	SBA	EUA	BSA	
	H. influenzae	HTM	EUB	BSBNAD						
Temperature (°C)	All	35	30	40		All	35	30	40	
Incubation time (h)	*S. aureus*, *E. coli*	20	16[a]	48		*S. aureus*, *E. coli*	20	16[a]	48	
	S. pneumoniae, *H. influenzae*	24	16	48		*S. pneumoniae*	24	16	48	
Inoculum	All	28×10^5 CFU/ml	10^4 CFU/ml	10^6 CFU/ml	10^7 CFU/ml	All	2×10^4 CFU/spot	2×10^3 CFU/spot	2×10^5 CFU/spot	2×10^6 CFU/spot
Atmosphere	All	Ambient	5% CO_2	10% CO_2		*S. aureus*, *E. coli*	Ambient	5% CO_2	10% CO_2	
						S. pneumoniae	5% CO_2	Ambient	10% CO_2	
pH	All	7.2–7.4	5.5[b]	6.5[b]	8.5[b]					
Calcium (μg/ml)	All	20–25	50	100						
Magnesium (μg/ml)[c]	All	10–12.5	+12.5	+25						
Zinc (mmol/liter)[c]	All	CAMHB	+2	+5	+10					
Potassium (mmol/liter)[c]	All	CAMHB	+12.5	+25	+50					
Thymidine (μg/ml)[c]	All	CAMHB	+1	+5						
% serum[d]	All	CAMHB	25[b]	50[b]						
Albumin (μg/dl)[d]	All	CAMHB	+4							
% polysorbate 80[e]	All	CAMHB	0.002							
% lung surfactant[f]	All	CAMHB	1	5						

BMD, broth microdilution; MD, macrodilution; AD, agar dilution; CAMHB, cation adjusted Mueller-Hinton broth; LHB, lysed horse blood (5%); HTM, Haemophilus test media; ISB, IsoSensitest broth; BSB, ISB–5% LHB; EUB, CAMHB–5% LHB–20 mg/liter NAD; BSB-NAD, ISB–5% LHB–20 mg/liter NAD; MHA, Mueller-Hinton agar; ISA, IsoSensitest agar; SBA, MHA–5% sheep blood agar; EUA, MHA–5% defibrinated horse blood–20 mg/liter NAD; BSA, MHA–5% defibrinated horse blood.

[a]The minimum incubation time for the reference CLSI BMD and AD methods is 16 h.

[b]The pH was adjusted to 5.5 and 6.5 with HCl and to 8.5 with NaOH.

[c]The indicated additional component was added to the media at the stated concentration(s); the final concentration in the media was not measured.

[d]Normal human pooled serum and human albumin (Innovative Research, Novi, MI) were added to the media at the stated concentrations.

[e]Polysorbate 80 (Sigma-Aldrich, St. Louis, MO) was added to the media at concentration of 0.002%.

[f]Surfactant (Survanta; Abbott Pharmaceuticals) was added to CAMHB to achieve the stated surfactant concentrations (19).

sterilized and added to the broth following autoclave sterilization of the broth. At the higher inoculum concentrations of 10^6 and 10^7 CFU/ml, although there was slight turbidity of the wells prior to incubation, MIC endpoints were easily determined on the basis of the presence of large buttons of growth following incubation.

Agar dilution. The effect of media was also determined for the AD method by using Mueller-Hinton agar (MHA; Becton Dickinson, Sparks, MD) and IsoSensitest agar (ISA; Oxoid, Basingstoke, United Kingdom) against *S. aureus* and *E. coli* and MHA–5% sheep blood (SBA), MHA–5% defibrinated horse blood (Cleveland Scientific, Bath, OH)–20 mg/liter NAD (EUA), and ISA–5% defibrinated horse blood (BSA) against *S. pneumoniae*. The following significant variables were studied for all study isolates by AD: temperature, incubation time, atmospheric condition, and inoculum concentration.

Data analysis. The mean doubling dilution difference of the MIC determined using each method (including AD, MD, media, and other MIC variables) from the reference method MIC (tested on the same day) and the essential agreement (percentage of the variable method MIC within ± 1 dilution of the reference method MIC) were calculated. The dilution difference was determined by converting the MIC to $\log_2 + 10$ values, and the reference value was subtracted from the variable method value to obtain the dilution difference (e.g., if reference MIC was 0.5 μg/ml [$\log_2 + 10 = 9$] and the variable method MIC was 1.0 μg/ml [$\log_2 + 10 = 10$], the dilution difference was 1 [$10 - 9$]). Mean dilution differences and

TABLE 5 Reference method and Variation of Disk Diffusion methods Studied

Parameter or condition	Disk diffusion (gepotidacin [10-μg] BD and MAST and linezolid [30-μg] BD disks) result						
	Test organism(s)	Reference method	Variables studied				
			1	2	3	4	5
Medium	*S. aureus*	MHA (BD)	MHA (Oxoid)	MHA (Hardy)	ISA (CLSI inoculum[a])	ISA (BSAC inoculum[b])	
	S. pneumoniae, *S. pyogenes*	SBA (BD)	SBA (Oxoid)	SBA (Hardy)	EUA	BSA (CLSI inoculum[b])	BSA (BSAC inoculum[b])
Temperature (°C)	All	35	30	40			
Incubation time (h)	*S. aureus*	16–18	14	24	48		
	S. pneumoniae, *S. pyogenes*	20–24	16	18	48		
Inoculum (CFU/ml)	All	10^8	10^6	10^7	10^9		
Atmosphere	*S. aureus*	Ambient	5% CO_2	10% CO_2			
	S. pneumoniae, *S. pyogenes*	5% CO_2	Ambient	10% CO_2			
pH	All	7.2–7.4	5.5[c]	6.5[c]	8.5[c]		
Calcium (μg/ml)	All	20–25	50	100			
Magnesium (μg/ml)[d]	All	10–12.5	+12.5	+25			
Zinc (mmol/liter)[d]	All	MHA	+2	+5	+10		
Potassium (mmol/liter)[d]	All	MHA	+12.5	+25	+50		
Thymidine (μg/ml)[d]	All	MHA	+1	+5			
% polysorbate 80[e]	All	MHA	0.002				

MHA, Mueller-Hinton agar; SBA, MHA–5% sheep blood agar; ISA, IsoSensitest agar; EUA, MHA–5% defibrinated horse blood–20 mg/liter NAD; BSA, MHA–5% defibrinated horse blood.

[a]The CLSI inoculum was a 0.5 McFarland standard.

[b]The BSAC inoculums used were 1:10 for *S. aureus* and *S. pneumoniae* (10^7 CFU/ml) and 1:100 for beta-hemolytic streptococci (10^6 CFU/ml).

[c]The pH was adjusted to 5.5 and 6.5 with HCl and to 8.5 with NaOH.

[d]The indicated additional component was added to the media at the stated concentration(s); the final concentration in the media was not measured.

[e]Polysorbate 80 (Sigma-Aldrich, St. Louis, MO) was added to the media at concentration of 0.002%.

essential agreement percentages for method variations that demonstrated <90% essential agreement compared to the reference method MIC determined on the same day of testing are shown in Tables 1 and 2. Mean dilution differences and essential agreement percentages are not shown for method variations that were in agreement with the reference method MIC (essential agreement, ≥90%).

Disk diffusion. Disk diffusion testing was performed against 30 *S. aureus* isolates (the same 10 strains tested in the MIC study and an additional 10 methicillin-susceptible and 10 methicillin-resistant strains), 15 *S. pneumoniae* isolates (the same 10 strains tested in the MIC study and an additional 2 penicillin-susceptible strains, 2 penicillin-intermediate strains, and 1 penicillin-resistant strain), and 15 *S. pyogenes* isolates (including 3 macrolide- and 3 tetracycline-resistant strains) according to CLSI methods with gepotidacin (10 μg) disks from 2 manufacturers (BD disks [Becton Dickinson, Sparks, MD] and MAST disks [Hardy Diagnostics, Santa Maria, CA]) and linezolid (30 μg) disks (Becton Dickinson, Sparks, MD) (17). All agar plates used in this study were prepared by the testing laboratory. The effect of media was determined by using Mueller-Hinton agar (MHA; Becton Dickinson, Sparks, MD; Oxoid, Basingstoke, United Kingdom; Hardy Diagnostics, Santa Maria, CA) and IsoSensitest agar (ISA; Oxoid, Basingstoke, United Kingdom) against *S. aureus* and MHA–5% sheep blood (SBA; MHA from Becton Dickinson, Sparks, MD, Oxoid, Basingstoke, United Kingdom, and Hardy Diagnostics, Santa Maria, CA, and sheep blood from Cleveland Scientific, Bath, OH), MHA–5% defibrinated horse blood (BSA; MHA from Becton Dickinson, Sparks, MD, and horse blood from Cleveland Scientific, Bath, OH), and BSA with addition of 20 mg/liter NAD (EUA; Sigma-Aldrich, St. Louis, MO) against *S. pneumoniae* and *S. pyogenes*. Quality control strains *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, and *S. pneumoniae* ATCC 49619 were tested each day for all reference and variable disk diffusion methods for all disks and compared to CLSI reference QC ranges (with the exception of *S. aureus* ATCC 29213, which was tested for informational purposes) (16). The following significant variables were studied: media, temperature, incubation time, atmospheric conditions, inoculum concentrations, and pH, calcium, magnesium, zinc, potassium, thymidine, and polysorbate 80 levels. All medium additives were filter sterilized and added to the agar following autoclave

sterilization of the agar. Data analysis included calculation of the difference in mean zone diameter for each variable method from the reference method mean zone diameter (tested on the same day) and calculation of essential agreement (percentage of variable method zone diameters within ±4 mm of the reference method zone diameters based on the investigator's criteria).

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