

## Influence of Human Serum on Pharmacodynamic Properties of an Investigational Glycopeptide, LY333328, and Comparator Agents against *Staphylococcus aureus*

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**The MICs and MBCs of 15 antibiotics for two strains of *Staphylococcus aureus* were determined in Mueller-Hinton broth (MHB) and 90% serum–10% MHB. Subsequent experiments established that highly protein-bound antibiotics ( $\geq 80\%$ ), such as LY333328, demonstrated higher MICs and MBCs, less killing over an 8-h interval, and shorter postantibiotic effects in 90% serum–10% MHB than in MHB alone. Albumin was demonstrated to be almost solely responsible for changes in the aforementioned pharmacodynamic parameters of LY333328.**

Currently, in vitro antibiotic susceptibility testing performed in standard microbiological media such as Mueller-Hinton broth (MHB) is used to determine appropriate therapy for patients with infectious diseases (12). The predictive value of antibiotic activity in microbiological media for in vivo drug efficacy has been established in many instances; however, detailed data describing the influence of biological fluids, such as human serum, on antibiotic activity are, at present, limited. Antibiotics vary in their affinity for binding to plasma proteins, and it is believed that only the free, unbound fraction of drug is available for antibacterial action (4). Increases in the MICs in serum of highly-bound cephalosporins such as ceftriaxone for *Staphylococcus aureus* (7), cefoperazone for *Pseudomonas aeruginosa* (8), and cefonicid for *S. aureus* (3) have been noted. The MICs and MBCs of teicoplanin, a highly bound glycopeptide, for *S. aureus* are increased, and killing over 24 h is decreased, when the bacteria are grown in Mueller-Hinton broth supplemented with human serum (1, 15). At the same time, there is evidence of potential factors in serum which enhance the antibacterial action of some newer cephalosporins against gram-negative bacilli (9, 10) and of fluoroquinolones against *S. aureus* (6). Our goal was to study the effect of serum on the MICs, MBCs, time-kill curves, and postantibiotic effects (PAEs) of the investigational glycopeptide LY333328 and comparator agents for *S. aureus*. We want not only to define the influence of human serum on the pharmacodynamics of LY333328 and comparator agents but also to determine the serum component(s) responsible for protein binding and the level of protein binding at which this influence becomes significant.

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One reference strain (ATCC 29213) and one clinical blood isolate (M709) of *S. aureus* were used. LY333328 (Lilly Research Laboratories, Indianapolis, Ind.), teicoplanin, vancomycin, amoxicillin/clavulanate, cefazolin, cefotaxime, ceftriaxone, cefuroxime, ciprofloxacin, clindamycin, cloxacillin, erythromy-

cin, gentamicin, imipenem, and rifampin were provided by their manufacturers. Drugs were selected on the basis of their clinical effectiveness against *S. aureus* infections, representation of different antibiotic classes, and degrees of protein binding. Stock solutions of these antibiotics were prepared from standard powders as described in National Committee for Clinical Laboratory Standards (NCCLS) guidelines (12).

MHB supplemented with cations (25  $\mu\text{g}$  of  $\text{CaCl}_2$  per ml, 12.5  $\mu\text{g}$  of  $\text{MgSO}_4$  per ml) was used as the control medium. Pooled human plasma was obtained from the Manitoba Red Cross and stored at  $-84^\circ\text{C}$ . Serum was prepared daily from plasma by recalcification with 2 ml of 1 M  $\text{CaCl}_2$  per 100 ml of plasma. A 90% serum–10% MHB (no cations added) (MHBS) medium was prepared, and the pH was adjusted to 7.3 to equal that of MHB. The MHBS was then filter (pore size, 0.45  $\mu\text{m}$ ) sterilized. Biochemical analysis showed similar osmolalities for the two media. A solution of MHB containing bovine serum albumin (MHBA) at a concentration equivalent to that of the 90% serum (35  $\mu\text{g}/\text{ml}$ ) and one containing bovine serum albumin (35  $\mu\text{g}/\text{ml}$ ) and human  $\alpha_1$ -acid glycoprotein (mean serum concentration, 0.9  $\mu\text{g}/\text{ml}$ ) were also prepared (12). Blood agar plates were used for colony counts.

MICs and MBCs were determined by using NCCLS broth macrodilution guidelines (12, 13). Antibiotic killing of bacteria over time was determined as previously described (13, 18), with viable colony counts (CFU per milliliter) occurring at 0, 1, 2, 3, 4, 6, 8, 12, and 24 h. *S. aureus* cultures were incubated at  $37^\circ\text{C}$  in a shaking water bath and exposed to antibiotic at a concentration equal to the previously determined MIC in MHB for that strain. Growth controls were run to ensure equivalent rates of growth in all media. PAEs were determined by the standard method (5, 18). After 2 h of exposure to an antibiotic at a concentration equal to the MIC in MHB, cultures were diluted 1:1,000 to remove the drug, and the time for the culture to replicate 1  $\log_{10}$  unit was determined. Cultures were incubated at  $37^\circ\text{C}$  in a shaking water bath, and colony counts were performed when bacteria were first introduced to the antibiotic, 2 h later (both before and after the 1:1,000 dilution), and every 0.5 h thereafter for a minimum of 4 h after the dilution. The PAE was calculated as previously described (18). All MIC, MBC, time-kill curve, and PAE determinations were per-

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TABLE 1. Antibiotic MIC and MBC determinations for *S. aureus* ATCC 29213

Antibiotic	% Protein bound	MIC ( $\mu\text{g/ml}$ ) in:		MBC ( $\mu\text{g/ml}$ ) in:	
		MHB	MHBS	MHB	MHBS
Gentamicin	5–10	0.5	0.5	1	1
Amoxicillin/clavulanate <sup>b</sup>	17/20	0.5/0.25	0.5/0.25	2/1	2/1
Imipenem	20	0.015	0.03	0.03	0.06
Vancomycin	30	1	1	4	4
Cefotaxime	35	2	4	4	4
Cefuroxime	35	2	2	4	4
Ciprofloxacin	40	0.5	0.5	1	1
Erythromycin	60	0.5	0.25	8	8
Rifampin	75	0.015	0.03	0.125	0.25
Cefazolin	80	0.5	2 <sup>a</sup>	1	4 <sup>a</sup>
Ceftriaxone	83–96	4	16 <sup>a</sup>	8	32 <sup>a</sup>
LY333328	>80 (rat)	0.5	4 <sup>a</sup>	0.5	4 <sup>a</sup>
Teicoplanin	90	0.5	2 <sup>a</sup>	0.5	2 <sup>a</sup>
Clindamycin	94	0.125	1 <sup>a</sup>	2	16 <sup>a</sup>
Cloxacillin	94	0.25	2 <sup>a</sup>	0.5	4 <sup>a</sup>

<sup>a</sup> This is a value that is significantly ( $P \leq 0.05$ ) larger in MHBS.

<sup>b</sup> Values for each drug in this combination are given.

formed a minimum of three times per strain on separate occasions.

MICs and MBCs were interpreted as described in NCCLS guidelines (1, 13), which state that a fourfold or greater difference in values is significant. Time-kill curves were analyzed at 0, 8, and 24 h by using repeated-measure analysis of variance, while PAEs were analyzed by factorial analysis of variance. Post hoc comparisons were then made by use of Scheffe's method.

MICs and MBCs for the reference strain of *S. aureus*, ATCC 29213, in MHB and MHBS are shown in Table 1 along with literature values for protein binding of each antibiotic in serum (2). The MICs and MBCs for the clinical strain M709 differed from those for strain ATCC 29213 by no more than a factor of 2, and repeat experiments with both strains showed no significant variation from the median values shown.

Antibiotics showing significant differences in MICs determined in MHB and MHBS were LY333328, cefazolin, ceftriaxone, teicoplanin, clindamycin, and cloxacillin (Table 1). Susceptibilities to these six antibiotics were then retested a minimum of three times in MHBA. The antibiotic MIC and MBC values, respectively, determined in MHBA were as follows: LY333328, 4 and 4  $\mu\text{g/ml}$ ; cefazolin, 1 and 2  $\mu\text{g/ml}$ ; ceftriaxone, 8 and 16  $\mu\text{g/ml}$ ; teicoplanin, 2 and 2  $\mu\text{g/ml}$ ; clindamycin, 0.25 and 2  $\mu\text{g/ml}$ ; and cloxacillin, 2 and 4  $\mu\text{g/ml}$ . Clindamycin MIC and MBC values were also determined in MHBA supplemented with  $\alpha_1$ -acid glycoprotein (0.9  $\mu\text{g/ml}$ ); they were 1 and 16  $\mu\text{g/ml}$ , respectively. The MICs and MBCs for the clinical strain M709 differed from those for strain ATCC 29213 by no more than a factor of 2, and repeat experiments with both strains showed no significant variation from the median values presented.

The MICs and MBCs of cefazolin in MHBS for both strains of *S. aureus* tested were fourfold higher than those in MHB, while both values determined in MHBA were twofold higher than those in MHB. Ceftriaxone MICs and MBCs were increased fourfold in MHBS relative to those in MHB, and when tested in MHBA, changes in ceftriaxone MICs and MBCs were both limited to twofold increases over those in MHB. Clindamycin and cloxacillin MICs and MBCs in MHBS were both increased eightfold over those in MHB. When MHBA was used, cloxacillin again showed results eightfold higher than

those in MHB. Clindamycin, however, showed no significant changes in its MICs and MBCs in MHBA compared to those in MHB until  $\alpha_1$ -acid glycoprotein (0.9  $\mu\text{g/ml}$ ) was added, at which point an eightfold increase such as that demonstrated with MHBS occurred.

Time-kill curve experiments were performed in MHB, MHBA, and MHBS with the eight antibiotics listed in Table 2. Bacterial killing following 8 h of incubation in MHBS and MHBA was negligible for LY333328, teicoplanin, cefazolin, ceftriaxone, clindamycin, cloxacillin, and vancomycin, while cultures incubated with ciprofloxacin demonstrated 1 log<sub>10</sub> kill at 8 h (Table 2). At 24 h of incubation, bacterial killing had been significantly reduced with all antibiotics in all three media tested except for cloxacillin in MHB (data not shown). The results for growth controls in all media showed no significant differences between each other.

Less killing of the two strains of *S. aureus* occurred ( $P < 0.001$ ) in MHBA and MHBS than in MHB, but there was no significant difference between killing in MHBA and that in MHBS by LY333328 (Fig. 1) and teicoplanin (data not shown). Similarly, no significant difference was noted between cloxacillin killing in MHBA and that in MHBS, but there was significantly less ( $P < 0.001$ ) killing in both of these media than in MHB. Ceftriaxone and cefazolin showed decreased killing ( $P < 0.001$ ) in MHBA and MHBS compared to that in MHB, but in addition a small but significant ( $P < 0.01$ ) decrease in killing was demonstrated by both antibiotics in MHBS compared to that in MHBA (data not shown). Clindamycin showed less killing ( $P < 0.001$ ) in MHBS than in MHBA and MHB, but there was no significant difference in killing by clindamycin between that in MHBA and that in MHB. However, when  $\alpha_1$ -acid glycoprotein (0.9  $\mu\text{g/ml}$ ) was added to MHBA, a decrease in killing ( $P < 0.001$ ) compared to that in MHB was noted, and the difference in killing by clindamycin between that in MHBA and that in MHBS disappeared. No significant differences in killing over 24 h between media were noted for either ciprofloxacin or vancomycin.

Mean results of PAE determinations of antibiotics for both strains of *S. aureus* in MHB, MHBA, and MHBS are shown in Table 3. In each case, PAE values of antibiotics for the reference and clinical strains showed no significant difference when compared to each other. Significantly shorter PAEs ( $P < 0.001$ ) were observed in both MHBA and MHBS than in MHB for LY333328, teicoplanin, cefazolin, ceftriaxone, cloxacillin, and clindamycin (but only when  $\alpha_1$ -acid glycoprotein [0.9  $\mu\text{g/ml}$ ] was added to the MHBA). In addition, no significant difference between PAEs in MHBA and MHBS was noted for these antibiotics. Clindamycin showed no significant decrease in PAE

TABLE 2. Killing of *S. aureus* ATCC 29213 by antibiotics at 1  $\times$  MIC following 8 h of incubation in three media<sup>a</sup>

Antibiotic	Killing (%) at 8 h in:		
	MHB	MHBS	MHBA
LY333328	99.9	0	5
Teicoplanin	10	0	0
Cefazolin	95	0	0
Ceftriaxone	95	0	0
Clindamycin	50	0	0 <sup>b</sup>
Cloxacillin	99.9	0	0
Vancomycin	20	20	15
Ciprofloxacin	99	90	90

<sup>a</sup> Killing represented by subtracting inoculum at 8 h from the initial inoculum; 0 represents no kill or net growth at 8 h.

<sup>b</sup> Result for MHBA plus  $\alpha_1$ -acid glycoprotein (0.9  $\mu\text{g/ml}$ ).

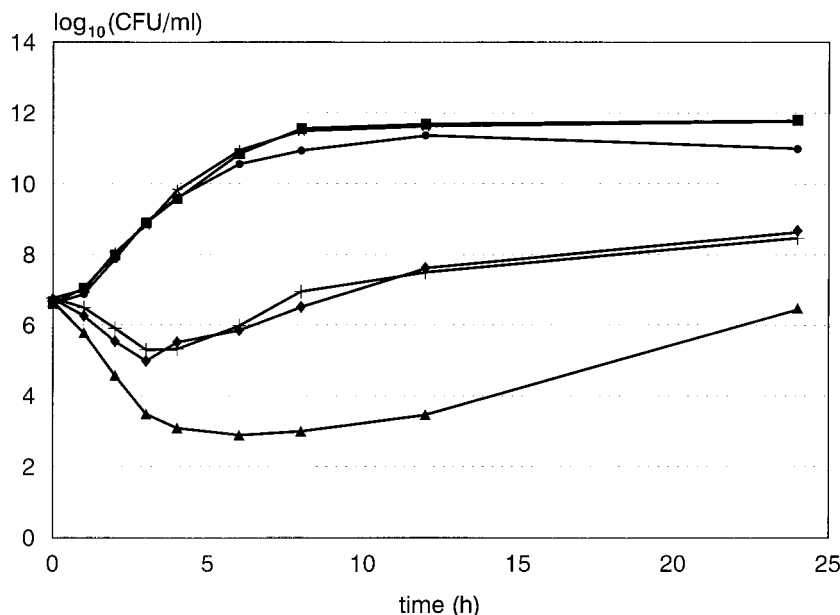


FIG. 1. Representative time-kill curve of ATCC 29213 *S. aureus* exposed to LY333328 at a concentration equal to the MIC in MHB (0.5 µg/ml). Symbols: ■, MHB control; ▲, MHB plus LY333328; ●, MHBA control; ◆, MHBA plus LY333328; ★, MHBS control; ✚, MHBS plus LY333328.

in MHBA without  $\alpha_1$ -acid glycoprotein as compared to that in MHB. Vancomycin had similar PAEs in all three media. The PAE of ciprofloxacin was significantly ( $P < 0.05$ ) longer in MHBS than in MHB and MHBA.

We observed that highly protein-bound antibiotics such as LY333328 possess decreased activity in human serum, decreased killing over time, and shorter PAEs. From our experiments, it appears that this reduction in activity is significant when the bound protein fraction of antibiotic is 80% or higher, as was the case with LY333328 (80% bound in rat), cefazolin (80%), ceftriaxone (83 to 96%), teicoplanin (90%), clindamycin (94%), and cloxacillin (94%). It should also be noted that further experiments showed that when antibiotic concentrations in MHBS were raised to their MICs in that medium (data not shown), the differences in killing over time and PAEs between MHB and MHBS were no longer significant (i.e., the higher total drug concentration in MHBS is believed to have resulted in a free drug concentration equivalent to that in MHB).

Albumin appears to be the primary binding protein for LY333328 and for the  $\beta$ -lactam antibiotics tested, as well as teicoplanin. The effect of MHBA on cloxacillin was virtually identical to that of MHBS in all experiments. With *S. aureus*, cefazolin and ceftriaxone showed significantly lower activity in MHBA than in MHB, but, unlike cloxacillin, their activities in MHBA and MHBS also showed some differences. The MICs of these antibiotics in MHBA were intermediate between those in MHB and MHBS, and although time-kill curves showed less killing in MHBA than in MHB, there was still more killing in MHBA than in MHBS. Antibiotic PAEs in MHBA were slightly longer than those in MHBS for cefazolin and ceftriaxone but not significantly so. No significant change in any of the results occurred when the albumin in the MHBA was increased to 50 µg/ml, when  $\alpha_1$ -acid glycoprotein (0.9 µg/ml) was added to the MHBA for antibiotics other than clindamycin, when the calcium and magnesium levels in the MHBA were adjusted to match the concentrations in MHBS, when the MHBS was heated for 30 min at 56°C to inactivate comple-

ment, or when human serum albumin was substituted for bovine serum albumin in the MHBA (data not shown). Stratton and Reller found similar discrepancies between human serum and a MHB-5% albumin solution for cefazolin and discovered that the bound fraction of antibiotic was higher in serum than in the albumin solution (16). It is thus possible that there are other minor proteins in serum that bind the cephalosporins cefazolin and ceftriaxone, although the identity of these proteins is uncertain. Sun et al. showed that ceftriaxone is capable of binding to immunoglobulin G in serum and that this binding could be particularly important at low total drug concentrations, such as those used in our experiments (17). The possibility also exists that factors present in MHB are occupying sites on the albumin in MHBA that the two cephalosporins would normally bind to, although it is highly unlikely that such factors would be present in sufficient amounts to have an appreciable effect. Clindamycin, a basic lincosamide, was shown to bind primarily with  $\alpha_1$ -acid glycoprotein, since when it was added to MHBA, the antibiotic activity was not significantly different from that in MHBS. Further studies showed that

TABLE 3. Mean PAE determinations for *S. aureus* ATCC 29213 and M709

Antibiotic	PAE (min [mean $\pm$ SD]) in:		
	MHB	MHBS	MHBA
LY333328	65 $\pm$ 7	5 $\pm$ 3	5 $\pm$ 3
Teicoplanin	83 $\pm$ 5	7 $\pm$ 5	9 $\pm$ 4
Cefazolin	48 $\pm$ 3	2 $\pm$ 2	7 $\pm$ 4
Ceftriaxone	56 $\pm$ 4	1 $\pm$ 3	15 $\pm$ 7
Clindamycin	88 $\pm$ 4	11 $\pm$ 4	73 $\pm$ 6 (15 $\pm$ 4) <sup>a</sup>
Cloxacillin	37 $\pm$ 3	1 $\pm$ 3	3 $\pm$ 3
Vancomycin	50 $\pm$ 4	50 $\pm$ 4	48 $\pm$ 5
Ciprofloxacin	60 $\pm$ 5	99 $\pm$ 4	58 $\pm$ 5

<sup>a</sup> Result for MHBA plus  $\alpha_1$ -acid glycoprotein (0.9 µg/ml) is shown in parentheses.

$\alpha_1$ -acid glycoprotein alone in MHB at physiologic concentrations also showed no significant difference from MHBS when clindamycin activity was being examined, providing further evidence that  $\alpha_1$ -acid glycoprotein, not albumin, is the main binding protein for this antibiotic.

Previous work by other investigators testing a single strain of methicillin-resistant *S. aureus* demonstrated results somewhat different from those reported here (11). The addition of albumin (40  $\mu$ g/ml) to MHB did not significantly alter LY333328 or vancomycin MICs but did result in four- and eightfold increases in MBCs, respectively. A medium containing 50% pooled human serum–50% MHB resulted in inconsistent LY333328 MICs and MBCs and did not significantly alter vancomycin MICs or MBCs. Interestingly, in a concurrent set of experiments performed by the same investigators, the addition of albumin (40  $\mu$ g/ml) to MHB resulted in an eightfold increase in the LY333328 MIC for a strain of vancomycin-resistant *Enterococcus faecium* (11). However, no significant changes in MIC and MBC determinations were noted when 50% pooled human serum–50% MHB was used. Investigators in this study were unable to detect the effect of proteins on a strain of methicillin-resistant *S. aureus* or antibiotic susceptibility (11).

The clinical relevance of antibiotic protein binding is a subject of debate (4). If all else was equal, one would likely select a less-protein-bound antibiotic over a highly bound one for therapy of infectious diseases. In fact, this is rarely the case, since different antibiotics are often quite dissimilar in both intrinsic antibacterial action and in pharmacokinetics and other factors usually take precedence in selection of appropriate antibacterial chemotherapy. Protein binding could be of considerable importance, however, if the antibiotic MIC for an infecting organism was shown to be close to the achievable drug concentration in serum. Although total antibiotic levels may be above the MIC, free drug levels may not, resulting in treatment failure. For infections such as bacterial meningitis or endocarditis, where antibiotic levels above the MBC are desired, protein binding of a potential agent could be even more critical. Indeed, Chambers et al. cite protein binding of cefonicid as the probable reason for its failure in a once-daily regimen for treatment of endocarditis (3).

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