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Effect of Protein Binding on the Pharmacological Activity of Highly Bound Antibiotics▽

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During antibiotic drug development, media are frequently spiked with either serum/plasma or protein supplements to evaluate the effect of protein binding. Usually, previously reported serum or plasma protein binding values are applied in the analysis. The aim of this study was to evaluate this approach by experimentally measuring free, unbound concentrations for antibiotics with reportedly high protein binding and their corresponding antimicrobial activities in media containing commonly used protein supplements. Free, unbound ceftriaxone and ertapenem concentrations were determined in bacterial growth medium with and without bovine/human serum albumin, as well as adult bovine serum and human plasma using in vitro microdialysis. The corresponding antimicrobial activity was determined in MIC and time-kill curve experiments using *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* ATCC 6303 as test strains. A semimechanistic maximum effect model was simultaneously fitted to the data and respective EC_{50} (concentration at half-maximum effect) values compared. Protein binding differed significantly for ceftriaxone ($P < 0.05$) between human plasma (76.8 ± 11.0%) and commercially available bovine (20.2 ± 8.3%) or human serum albumin (56.9 ± 16.6%). Similar results were obtained for ertapenem (human plasma, 73.8 ± 11.6%; bovine serum albumin, 12.4 ± 4.8%; human serum albumin, 17.8 ± 11.5%). The MICs and EC_{50}s of both strains were significantly increased ($P < 0.05$) for ceftriaxone when comparing human and bovine serum albumin, whereas the EC_{50}s were not significantly different for ertapenem. Free, unbound antibiotic concentrations differed substantially between plasma and protein supplements and correlated well with antimicrobial efficacy. Therefore, free, active concentrations should be measured in the test system instead of correcting for literature protein binding values.

Most drugs bind to proteins or other biological materials, such as albumin; α_1-acid glycoprotein; lipoproteins; α-, β-, and γ-globulins; and erythrocytes. Thus, free, unbound drug concentrations in plasma decrease as the degree of binding to these compounds increases. It is a well-recognized fact that, at least for small molecules, only free, unbound drug distributes into the extravascular space and is responsible for pharmacological activity and/or side effects (22, 26, 40). For antibiotics in particular, reduced free, active drug concentrations as a result of protein binding are reflected in decreased antimicrobial activity (22). In theory, this effect is most pronounced for antibiotics with extensive protein binding. To date, most studies evaluating the effect of protein binding on the potency of an antibiotic against a certain pathogen determine changes in the respective MIC (22). Whereas both bacterial growth and killing are dynamic processes, the MIC is a static, highly variable threshold value, incapable of predicting antibiotic activity at concentrations apart from the MIC (19, 27). In comparison, the evaluation of growth and antibiotic-induced kill profiles over time (time-kill curves) provides more detailed information than the MIC. Once experimentally determined, time-kill curves can be characterized by the simultaneous fit of appropriate mathematical models and quantitatively compared by respective outcome parameters, such as the concentration at half-maximum effect (EC_{50}) (27). Time-kill curves have, consequently, been suggested as an experimental method for the evaluation of protein binding effects on antimicrobial activity (28, 40). In order to account for protein binding in these experiments, bacterial media are spiked with either human serum or protein supplements. When supplementing with human serum, its actual content frequently has to be limited to ≤50%, since it may inhibit bacterial growth or modify the antibacterial activity (13, 16, 22). On the other hand, when supplementing with proteins, usually human serum albumin (HSA) or comparatively less-expensive animal albumins (3, 9, 11) are employed, as HSA is the main natural binding component of antibiotics (15, 25, 33, 36, 40). Nevertheless, the actual free, unbound antibiotic concentration after either human serum or protein supplementation is rarely determined. Instead, literature or protein binding values determined in vitro are frequently employed to estimate free, unbound concentrations (5, 14).

The goal of the present study was to evaluate this approach by linking measured free, unbound concentrations of the two highly bound β-lactams ceftriaxone (83 to 96%) (30, 31, 38) and ertapenem (84 to 96%) (4) to their respective

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antimicrobial activity against gram-positive *Streptococcus pneumoniae* ATCC 6303 and gram-negative *Escherichia coli* ATCC 25922.

MATERIALS AND METHODS

Organisms. *E. coli* ATCC 25922 and penicillin-sensitive *S. pneumoniae* ATCC 6303 were obtained from the clinical microbiology laboratory at Shands Hospital at the University of Florida. *E. coli* and *S. pneumoniae* were grown in a CO_2 incubator (Barnstead-Thermolyne, Melrose Park, IL) in Mueller-Hinton broth (MHB) or Todd-Hewitt broth (THB) plus 5% CO_2, respectively. To ensure the purity of the bacterial strains, they were subcultured at least three times before either usage or freezing of the stock cultures. The bacterial inoculum was prepared from colonies incubated overnight on 5% sheep blood agar plates (Remel Microbiology Products, Lenexa, KS). The microorganisms were suspended in 0.9% sterile saline solution to a concentration equivalent to a 0.5 value in the McFarland scale (Remel Microbiology Products, Lenexa, KS) with a turbidimeter (A-JUST; Abbott Laboratories, North Chicago, IL). This value on the McFarland scale of 0.5 is equivalent to a number of 1×10^8 viable CFU/ml. Further dilution steps to reach a final working inoculum of approximately 5×10^5 CFU/ml were performed in broth.

Antibiotics and growth media. Ceftriaxone disodium was purchased from Sigma-Aldrich (St. Louis, MO), and ertapenem sodium was obtained from Merck & Co., Inc. (Whitehouse Station, NJ). The antibiotics were prepared and stored according to the manufacturer's recommendations. MHB (Becton Dickinson, Franklin Lakes, NJ) and THB (Difco, Detroit, MI) were used as liquid growth media. MHB and THB were both prepared according to the manufacturer's instructions and autoclaved prior to use at 121°C (15 min per 1 liter). The broth media were supplemented with either 40 g/liter bovine serum albumin (BSA) (catalog no. A3059, lot. no. 036K0735; Sigma-Aldrich, St. Louis, MO) or 40 g/liter HSA (catalog no. 12666, lot no. B75308-01; Calbiochem, La Jolla, CA) and filtered through 0.2-μm filters (Millipore, Billerica, MA) and the pH was adjusted to 7.4.

HPLC analysis. (i) Instrumentation. The high-performance liquid chromatography (HPLC) system for both ceftriaxone and ertapenem consisted of the Agilent 1100 series (Agilent Technologies, Waldbronn, Germany), comprising a model G1313 autosampler, a model G1311 quaternary pump and G1315 DAD UV detector, an Agilent Chemstation for LC systems, and a LiChrospher 100 reversed-phase 18 analytical column (RP-18 [5-μm particle size]; Merck KGaA, Darmstadt, Germany).

(ii) Chromatographic conditions. *(a) Ceftriaxone*. The ion-pair chromatography assay procedure was adapted from Kovar et al. (12). Reversed-phase HPLC was performed at room temperature at a flow rate of 1.0 ml/min. The mobile phase consisted of a mixture of buffer (7.5 mM KH_2PO_4; J. T. Baker Chemical Co., Phillipsburg, NY) and acetonitrile (56:44, vol/vol) with 5 mM hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO) as the ion-pair reagent. The final pH of the mobile phase was adjusted to 8.8. Twenty-five microliters of ceftriaxone were injected and detected at 280 nm. The run time was set to 16 min.

(b) Ertapenem. The chromatography procedure was adapted from Gordien et al. (8). The mobile phase consisted of 10 mM phosphate buffer adjusted to pH 6.5 with concentrated orthophosphoric acid and mixed with acetonitrile. A gradient was run at a flow rate of 1 ml/min. Forty microliters of ertapenem were injected and detected at 305 nm. The run time was set to 14 min.

Protein binding. Free, protein-unbound ceftriaxone and ertapenem concentrations were determined in in vitro dose-ranging (20, 40, 80, 160, and 320 μg/ml) extraction efficiency microdialysis experiments. Briefly, blank lactated Ringer (LR; Baxter Health Care, Deerfield, IL) solution is pumped through a flexible CMA 60 microdialysis probe (CMA Microdialysis AB, Solna, Sweden) at a constant flow rate of 2.0 μl/min. The relative recovery of the microdialysis probes was determined in LR only and calculated according to the equation $C(LR)_{dialysate}/C(LR)_{sample}$, where $C(LR)_{dialysate}$ is the free concentration recovered from the microdialysis probe and $C(LR)_{sample}$ is the concentration in the test tube.

Once the relative recovery was determined, free, unbound ceftriaxone and ertapenem concentrations were determined in triplicate at 37°C in THB, THB with BSA, THB with HSA, and pooled adult bovine serum (ABS; HyClone, Logan, UT) or pooled human plasma (HP; Shands Hospital at the University of Florida, Gainesville, FL). It was previously reported that serum binding properties can be altered by heat treatment (37). Therefore, untreated pooled HP was used for the in vitro protein binding experiments.

Protein binding (%) of the respective samples can then be calculated according to the equation $1 - C(sample)_{dialysate}/[RR \times C(sample)_{total}]$, where $C(sample)_{dialysate}$ is the concentration in the protein-free dialysate and $C(sample)_{total}$ is the mean concentration of the samples that were collected directly out of the test tube at the beginning and the end of the 30-min sampling period. The collected samples were analyzed by the reversed-phase HPLC methods described above.

Protein binding differences within the treatment groups were evaluated using analysis of variance followed by least-square means to test for pair-wise differences. All statistical analysis was performed in SAS 9.1.3 (SAS Institute Inc., Cary, NC). A P value of <0.05 was considered statistically significant.

Pharmacodynamics. (i) MIC. The MICs of *E. coli* ATCC 25922 and *S. pneumoniae* ATCC 6303 against ceftriaxone and ertapenem were determined six times according to the CLSI guidelines both in the presence and in the absence of BSA and HSA using a serial dilution twofold broth macrodilution method (34).

(ii) Time-kill curves. In vitro 6-h constant concentration time-kill curves were performed in triplicate for both ceftriaxone and ertapenem against the test strains in the presence and absence of BSA and HSA (34). Eight 50-ml cell culture flasks (Nunc; Nunc A/S, Roskilde, Denmark) were filled with 20 ml of bacteria-containing growth medium and incubated for 2 h before adding the antibiotic. The selection of respective ceftriaxone or ertapenem concentrations (Table 1) was based on their determined MICs and covered the entire antimicrobial spectrum, including the minimum inhibition of bacterial growth (0.25×, 0.5×, and 1× MIC), efficient bacterial killing (2× and 4× MIC), and maximum bacterial killing (8× and 16× MIC). Samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h. Bacterial counts were determined, using an adapted droplet-plate method (34). A control experiment with bacteria and no drug was run simultaneously. After incubation at 37°C for 20 to 24 h, viable counts were determined on all readable plates.

Mathematical modeling. A modified susceptibility-based two-compartment model was simultaneously fit to the time-kill curve data from experiments with and without BSA and HSA (21, 34). In this model, the overall change in the experimentally determined total number of bacteria was defined as the sum of self-replicating, antibiotic-sensitive cells and metabolically inactive, insusceptible persister cells (1). While bacterial growth could be sufficiently described by the growth-rate constant k_s (h^{-1}), antibiotic-induced kill was characterized by the maximum kill-rate constant k_{max} (h^{-1}), the antibiotic concentration C (μg/ml), and the EC_{50} (μg/ml). The final shape of the curve fit could be optimized by a Hill or shape factor (h).

In order to compare the respective EC_{50}s in the presence or absence of BSA or HSA, k_s, k_{max}, and h values were fitted across all treatment groups for each antibiotic and strain. Initial parameter estimates for k_s, k_{max}, and h were obtained from individual curve fits and compared for differences. Since no differences in the parameter estimates were observed (data not shown), arithmetic means were used as the initial estimates for the simultaneous curve fits across the treatment groups.

After simultaneously fitting the susceptibility-based sigmoidal maximum effect model to the experimental time-kill curve data with the nonlinear least-square regression software Scientist 3.0 (Micromath, Salt Lake City, UT), models were characterized by model selection criteria and graphs visually inspected for quality of fit. EC_{50} comparisons were done using analysis of variance, followed by least-square means. A P value of <0.05 was considered statistically significant.

RESULTS

Protein binding. As shown in Fig. 1, mean protein binding (± standard deviation) values for both ceftriaxone (76.8 ± 11.0%) and ertapenem (73.8 ± 11.6%) were higher in pooled HP than in THB with and without BSA or HSA, as well as pooled ABS ($P < 0.05$). For ceftriaxone, protein binding was significantly lower in commercially available BSA (20.2 ± 8.3%) and HSA (56.9 ± 16.6%), as well as pooled ABS (30.7 ± 6.2%). Similar lower protein binding values were also observed for ertapenem (BSA, 12.4 ± 4.8%; HSA, 17.8 ± 11.5%; pooled ABS, 38.3 ± 9.8%).

Pharmacodynamics. (i) MICs. The determined MICs (presented as modes) are shown in Table 1. No differences in the MICs were found for ertapenem against *S. pneumoniae* both in the presence and in the absence of BSA and HSA. However, in the presence of HSA (versus no albumin), MICs were in-

TABLE 1. Determined MICs (presented as modes) and simultaneously fitted model parameters (± standard deviations) of ceftriaxone and ertapenem against *E. coli* and *S. pneumoniae* in the presence and absence of BSA and HSA

Antibiotic, strain, and protein supplement[a]	Parameter					
	MIC (μg/ml)	k_s (h^{-1})	k_{max} (h^{-1})	EC_{50} (μg/ml)	h	MSC
Ceftriaxone						
E. coli ATCC 25922						
Without albumin	0.064	2.40 (±0.34)	6.34 (±0.13)	0.027[d] (±0.0007)	1.61 (±0.10)	4.24
With BSA	0.064			0.057[d] (±0.007)		
With HSA	2			1.096[b,c] (±0.0272)		
S. pneumoniae ATCC 6303						
Without albumin	0.01	1.56 (±0.08)	3.59 (±0.11)	0.004[d] (±0.0004)	2.18 (±0.14)	4.04
With BSA	0.02			0.015[d] (±0.0099)		
With HSA	0.16			0.084[b,c] (±0.0147)		
Ertapenem						
E. coli ATCC 25922						
Without albumin	0.015	2.76 (±0.89)	6.02 (±1.00)	0.010 (±0.0035)	2.54 (±0.14)	4.71
With BSA	0.015			0.020 (±0.0029)		
With HSA	0.03			0.014 (±0.0046)		
S. pneumoniae ATCC 6303						
Without albumin	0.025	2.28 (±0.13)	3.28 (±0.11)	0.010 (±0.0019)	2.65 (±0.25)	2.67
With BSA	0.025			0.010 (±0.0015)		
With HSA	0.025			0.019 (±0.0101)		

[a] BSA and HSA were added at a protein concentration of 40 g/liter.
[b] Significant difference ($P \leq 0.05$) from the value for the control (protein-free).
[c] Significant difference ($P \leq 0.05$) from the value for the sample with BSA.
[d] Significant difference ($P \leq 0.05$) from the value for the sample with HSA.

creased for ceftriaxone against *E. coli* and *S. pneumoniae*, as well as for ertapenem against *E. coli*. In comparison, when supplementing with BSA (versus no albumin), no changes in the MICs were detected for ceftriaxone against *E. coli* and ertapenem against *E. coli*, whereas values were increased for ceftriaxone against *S. pneumoniae*.

(ii) Time-kill curves. Qualitative evaluation of the time-kill curves showed that, in comparison to albumin-free medium, there are no differences in the growth of both *E. coli* and *S. pneumoniae* in the presence of BSA and HSA (Fig. 2). However, further evaluation showed differences in antimicrobial activity when comparing samples with and without albumin supplementation. These differences were most apparent at concentrations of 8× MIC (with respect to albumin-free medium). While the maximum kill effect was not reached at 8× MIC for ceftriaxone when HSA was added, these concentrations were sufficient for ertapenem and ceftriaxone in the presence of BSA as well as for ertapenem supplementing with HSA.

Mathematical modeling. Simultaneous curve fits of ceftriaxone and ertapenem against *E. coli* and *S. pneumoniae* with and without BSA or HSA are shown in Fig. 3 and 4. The corresponding model parameters for ceftriaxone and ertapenem against both strains in the presence and absence of BSA and HSA are listed in Table 1. For ceftriaxone, the calculated EC_{50}s for both strains were higher in the presence of HSA than in the presence of BSA ($P < 0.05$) or in the absence of albumin ($P < 0.05$). The difference remained significant after adjusting for multiple comparisons. In contrast, no differences in the

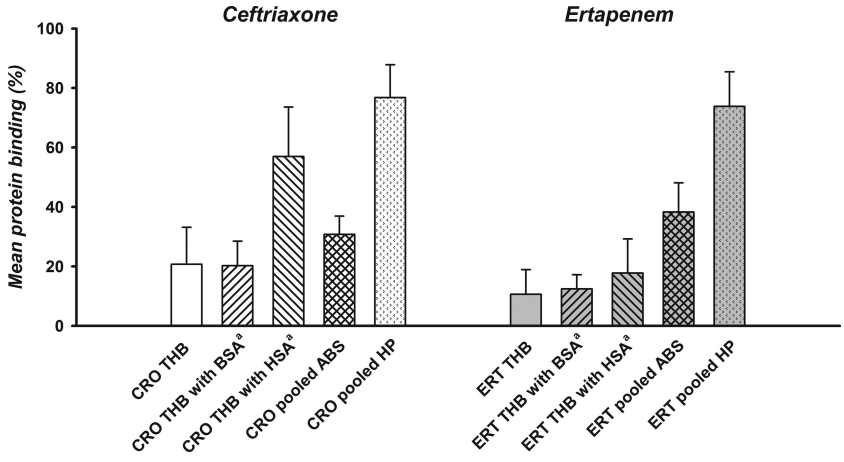

FIG. 1. In vitro mean protein binding (%) of ceftriaxone (CRO; white) and ertapenem (ERT; gray) in THB, THB with BSA (at a protein concentration of 40 g/liter), THB with HSA (at a protein concentration of 40 g/liter), pooled ABS, and pooled HP.

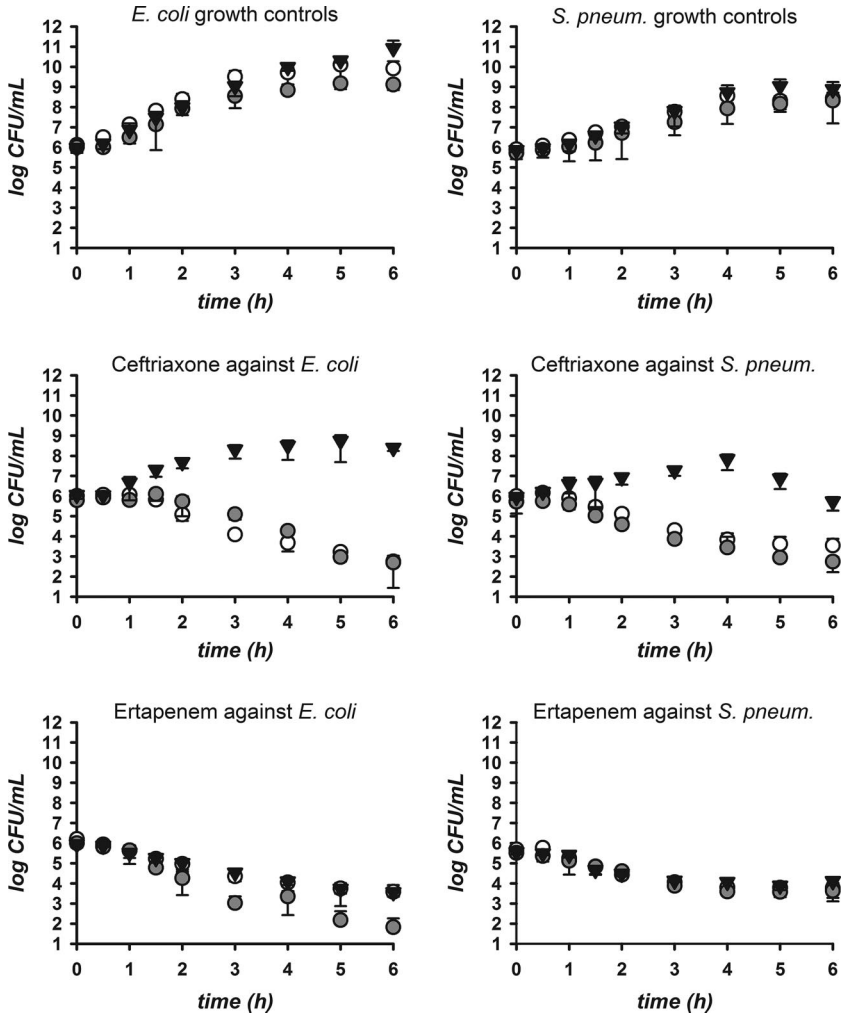

FIG. 2. Effect of BSA and HSA on bacterial growth and the maximum kill rate determined at 8× MIC (with respect to albumin-free medium) for ceftriaxone and ertapenem against *E. coli* and *S. pneumoniae* without (○) and with BSA (●) or HSA (▼), both at a protein concentration of 40 g/liter.

EC_{50}s were determined for ertapenem against both strains when it was supplemented with HSA compared to without albumin and with BSA.

DISCUSSION

The clinical significance of protein binding on antimicrobial activity continues to be controversial due to conflicting reports from in vitro MIC and/or time-kill curve experiments (29). In these experiments, bacterial media are spiked with either serum or protein supplements in order to produce and modify protein binding. However, whether the degree of drug binding in these in vitro test systems is representative of the respective physiological conditions is often unclear, since free, unbound concentrations are frequently not experimentally determined. Instead, reported literature values are commonly used to correct total concentrations for protein binding (5, 14). The results of our study, however, clearly show that this approach can be extremely misleading.

In this study, the MICs and constant concentration time-kill curves of two β-lactams with reportedly high protein binding, ceftriaxone (83 to 96%) (30, 31, 38) and ertapenem (84 to 96%) (4), were determined in the presence and absence of BSA and HSA (40 g/liter) and the outcomes compared. The results indicate that the antimicrobial activity of both ceftriaxone and ertapenem was decreased against both test strains, except for ertapenem against *S. pneumoniae*, in the presence of HSA. However, the MICs remained unchanged, except for ceftriaxone against *S. pneumoniae*, in the presence of BSA. In theory, these differences in pharmacodynamic outcome could be explained by albumin-induced effects on growth and maximum kill rates, as well as changes in potency (EC_{50}) (20, 22, 28). The evaluation of bacterial growth is, thereby, of particular importance since only dividing cells are susceptible to β-lactams (22). However, evaluation of the respective growth controls over time revealed that there were no significant differences in the growth rates of both test strains in the presence or absence of BSA and HSA (Fig. 2).

Once antibiotic is added, antimicrobial agents kill bacteria more rapidly as concentrations increase (23). Yet, at concen-

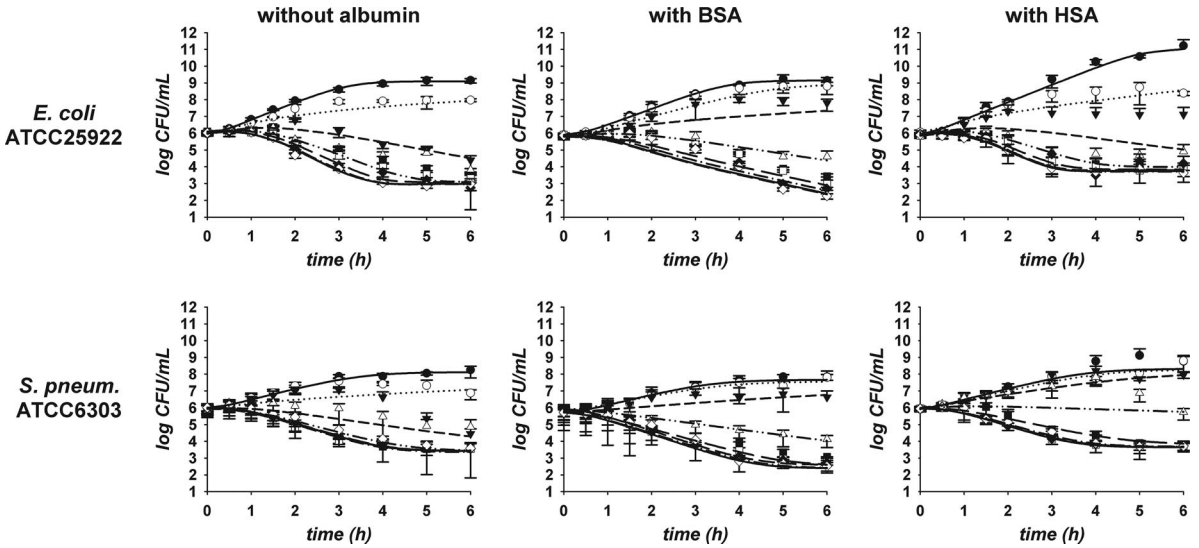

FIG. 3. Simultaneous curve fits for ceftriaxone against *E. coli* and *S. pneumoniae* in the presence and absence of BSA and HSA (both at a protein concentration of 40 g/liter) at concentrations of 0.25× MIC (----○----), 0.5× MIC (— ▼ —), 1× MIC (— ··· △ ··· —), 2× MIC (— ■ —), 4× MIC (— ·□· —), 8× MIC (— ◆ —), and 16× MIC (—◇—) plus growth controls (—●—). Symbols represent the experimental data; solid lines represent the simultaneous curve fits based on the pharmacokinetic/pharmacodynamic model.

trations of two to four times the MIC, the respective response varies (23). For β-lactams, the maximum kill effect is already observed. At this point, a further increase in antibiotic concentrations does not result in increased killing and the kill rate remains constant. The findings of our study are in agreement with these concepts. However, the results further revealed that ceftriaxone concentrations of 8× MIC (with respect to no albumin) are not high enough to reach k_{max} when HSA is added but are sufficient when BSA is present (Fig. 2). When further increasing concentrations to 16× to 32× MIC, the maximum kill effect was reached and no albumin-related effects on k_{max} were determined. In contrast, saturation in kill was achieved for ceftriaxone and ertapenem in the presence of BSA as well as for ertapenem when adding HSA at 8× MIC.

Nevertheless, substantial differences in the mean EC_{50}s were determined for ceftriaxone, whereas no significant differences in the mean EC_{50}s were determined for ertapenem in the presence of HSA. In comparison, no differences in the mean EC_{50}s were determined for both strains and antibiotics when they were supplemented with BSA. At this point, it is important to realize that in isolation, the results of the MIC and time-kill curves lead to ambiguous conclusions about the im-

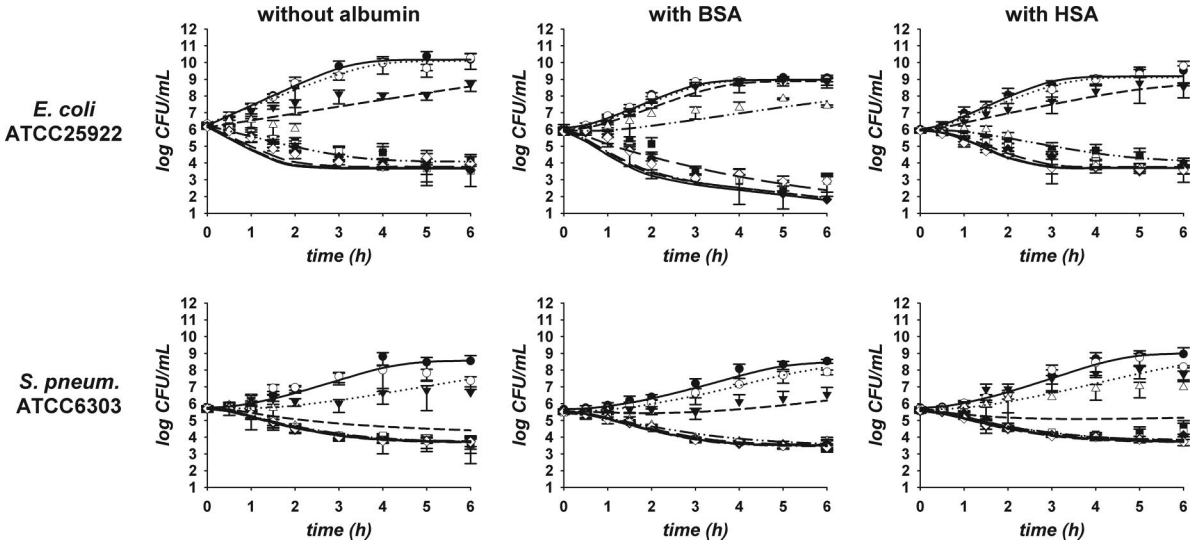

FIG. 4. Simultaneous curve fits for ertapenem against *E. coli* and *S. pneumoniae* in the presence and absence of BSA and HSA (both at a protein concentration of 40 g/liter) at concentrations of 0.25× MIC (----○----), 0.5× MIC (— ▼ —), 1× MIC (— ··· △ ··· —), 2× MIC (— ■ —), 4× MIC (— ·□· —), 8× MIC (— ◆ —), and 16× MIC (—◇—) plus a growth control (—●—). Symbols represent the experimental data; solid lines represent the simultaneous curve fits based on the pharmacokinetic/pharmacodynamic model.

pact of protein binding on antimicrobial activity. While a twofold increase in the MIC was observed for both ceftriaxone against *S. pneumoniae* in the presence of BSA and ertapenem against *E. coli* in the presence of HSA, no significant differences in the EC_{50}s were determined in the respective time-kill curve experiments. These differences in pharmacological outcome (MIC versus EC_{50}) are frequently attributed to the immanent high variability of the employed broth macrodilution (twofold) method (20, 27). Nevertheless, the fact that there are tremendous differences between the MICs and EC_{50}s of both ceftriaxone and ertapenem against both strains cannot simply be explained by variability or calculated free concentrations, since both antibiotics have very similarly reported protein binding values (ceftriaxone, 83 to 96%; ertapenem, 84 to 96%) (4, 30, 31, 38). However, the major assumption that the reported in vivo protein binding values reflect also the binding conditions in the in vitro system is rarely validated by experimentally measuring free, unbound concentrations.

Different methods, such as equilibrium dialysis, ultrafiltration, microdialysis, etc., have been used for the determination of protein binding and have shown comparable outcomes (2, 10). In our study, free, unbound concentrations were measured by in vitro microdialysis and respective protein binding values were calculated (Fig. 1). The results indicate that the observed differences in the antimicrobial activities of ceftriaxone and ertapenem can be explained by differences in their in vitro binding to the respective albumin supplements. While ceftriaxone is extensively bound to the tested HSA, it shows only little binding to BSA, and ertapenem hardly binds to either one. The findings of our in vitro microdialysis study are in agreement with those from Nix et al., where the binding of ertapenem to purified albumins was determined by ultrafiltration (22). The findings of both studies concurrently indicate that the in vitro binding to albumin supplements was substantially lower than previously reported literature protein binding values. The results of the ultrafiltration experiment further showed that the binding of ertapenem to various albumin supplements was greatly dependent on the albumin preparations used and differed substantially between suppliers (22). These observations may be explained by the lack of fatty acids in the albumin supplements (32) and/or the use of rigorous conditions, such as heat treatment or organic solvents during the purification process that can result in conformational changes in the respective albumin binding sites and, subsequently, reduced protein binding values (22). On the other hand, the significantly lower binding to pooled ABS, compared to pooled HP, indicates that for both antibiotics, the differences in binding capacities between species may play a role. For ceftriaxone, similar protein binding values have been previously determined in human, rat, baboon, and rabbit plasma, whereas substantially lower binding values were measured in dog plasma (24). This article by Popick et al. further revealed that the initially high protein binding of ceftriaxone (90 to 95%) at concentrations of <100 μg/ml is considerably decreased to approximately 60% at higher concentrations (>400 μg/ml) (24). In our study, mean protein binding values (ceftriaxone, $76.8 \pm 11.0\%$) were determined at concentrations ranging from 20 to 320 μg/ml and are in agreement with the previously reported range.

At this point, it also should be mentioned that there are other factors that may provide an explanation for the differences in the reported outcomes that range from no protein binding effects (7, 14) and delay in the onset of activity (5, 6) to only free, unbound drug being responsible for the antimicrobial activity (18, 22, 26, 35, 39, 40). For example, it has been shown that the bacterial density and the state of nutrition are crucial parameters and usually differ in the MIC and time-kill curve studies from the physiological conditions. While a lack of nutrition and low bacterial numbers hinder the experimental conduct, very high initial inoculum sizes seem to alter the susceptibility toward antimicrobial agents and might mask protein binding effects (17). It would, therefore, be important to internationally standardize the methodology of protein binding studies in order to minimize the experimental bias.

In conclusion, the results of the constant and changing concentration experiments clearly show that protein binding reduces in vitro antimicrobial activity. The study results further demonstrate that binding to commercially available protein supplements can substantially differ from that of serum or plasma and greatly depends on the supplement used. Correcting total concentrations for the reported literature binding values is consequently unreliable. Instead, free, active antibiotic concentrations should be experimentally measured in the actual test system. In vitro microdialysis is a convenient sampling tool for this purpose. In addition, an international standardization of the respective test systems might help prevent further misinterpretation of the impact of protein binding on antimicrobial activity.

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REFERENCES

1. **Balaban, N. Q., J. Merrin, R. Chait, L. Kowalik, and S. Leibler.** 2004. Bacterial persistence as a phenotypic switch. Science **305:**1622–1625.
2. **Barre, J., J. M. Chamouard, G. Houin, and J. P. Tillement.** 1985. Equilibrium dialysis, ultrafiltration, and ultracentrifugation compared for determining the plasma-protein-binding characteristics of valproic acid. Clin. Chem. **31:**60–64.
3. **Boswell, F. J., J. P. Ashby, J. M. Andrews, and R. Wise.** 2002. Effect of protein binding on the in vitro activity and pharmacodynamics of faropenem. J. Antimicrob. Chemother. **50:**525–532.
4. **Burkhardt, O., M. Brunner, S. Schmidt, M. Grant, Y. Tang, and H. Derendorf.** 2006. Penetration of ertapenem into skeletal muscle and subcutaneous adipose tissue in healthy volunteers measured by in vivo microdialysis. J. Antimicrob. Chemother. **58:**632–636.
5. **Cafini, F., L. Aguilar, N. Gonzalez, M. J. Gimenez, M. Torrico, L. Alou, D. Sevillano, P. Vallejo, and J. Prieto.** 2007. In vitro effect of the presence of human albumin or human serum on the bactericidal activity of daptomycin against strains with the main resistance phenotypes in Gram-positives. J. Antimicrob. Chemother. **59:**1185–1189.
6. **Cha, R., and M. J. Rybak.** 2004. Influence of protein binding under controlled conditions on the bactericidal activity of daptomycin in an in vitro pharmacodynamic model. J. Antimicrob. Chemother. **54:**259–262.
7. **Fontana, R., M. Aldegheri, M. Ligozzi, G. Lo Cascio, and G. Cornaglia.** 1998. Interaction of ceftriaxone with penicillin-binding proteins of Escherichia coli in the presence of human serum albumin. J. Antimicrob. Chemother. **42:**95–98.
8. **Gordien, J. B., E. Boselli, C. Fleureau, B. Allaouchiche, G. Janvier, O. Lalaude, M. C. Saux, and D. Breilh.** 2006. Determination of free ertapenem in plasma and bronchoalveolar lavage by high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. B **830:**218–223.
9. **Heinze, A., and U. Holzgrabe.** 2006. Determination of the extent of protein binding of antibiotics by means of an automated continuous ultrafiltration method. Int. J. Pharm. **311:**108–112.
10. **Herrera, A. M., D. O. Scott, and C. E. Lunte.** 1990. Microdialysis sampling for determination of plasma protein binding of drugs. Pharm. Res. **7:**1077–1081.
11. **Huang, Y., and Z. Zhang.** 2004. Binding study of drug with bovine serum

album [sic] using a combined technique of microdialysis with flow-injection chemiluminescent detection. J. Pharm. Biomed. Anal. **35:**1293–1299.
12. **Kovar, A., T. Dalla Costa, and H. Derendorf.** 1997. Comparison of plasma and free tissue levels of ceftriaxone in rats by microdialysis. J. Pharm. Sci. **86:**52–56.
13. **Lang, S. D., G. L. Cameron, and P. R. Mullins.** 1981. Anomalous effect of serum on the antimicrobial activity of cefoperazone. Drugs **22**(Suppl. 1)**:**52–59.
14. **Laue, H., T. Valensise, A. Seguin, S. Hawser, S. Lociuro, and K. Islam.** 2007. Effect of human plasma on the antimicrobial activity of iclaprim in vitro. J. Antimicrob. Chemother. **60:**1388–1390.
15. **Lee, B. L., M. Sachdeva, and H. F. Chambers.** 1991. Effect of protein binding of daptomycin on MIC and antibacterial activity. Antimicrob. Agents Chemother. **35:**2505–2508.
16. **Leggett, J. E., and W. A. Craig.** 1989. Enhancing effect of serum ultrafiltrate on the activity of cephalosporins against gram-negative bacilli. Antimicrob. Agents Chemother. **33:**35–40.
17. **MacGowan, A. P., K. E. Bowker, C. M. Tobin, A. R. Noel, A. M. Lovering, and A. H. Thomson.** 2008. Pharmacodynamics of vancomycin against MRSA: implications of bacterial inoculum for clinical breakpoints, abstr. P1242. 18th Eur. Congr. Clin. Microbiol. Infect. Dis., Barcelona, Spain.
18. **Merrikin, D. J., J. Briant, and G. N. Rolinson.** 1983. Effect of protein binding on antibiotic activity in vivo. J. Antimicrob. Chemother. **11:**233–238.
19. **Mouton, J. W., M. N. Dudley, O. Cars, H. Derendorf, and G. L. Drusano.** 2005. Standardization of pharmacokinetic/pharmacodynamic (PK/PD) terminology for anti-infective drugs: an update. J. Antimicrob. Chemother. **55:**601–607.
20. **Mueller, M., A. de la Pena, and H. Derendorf.** 2004. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. Antimicrob. Agents Chemother. **48:**369–377.
21. **Nielsen, E. I., A. Viberg, E. Lowdin, O. Cars, M. O. Karlsson, and M. Sandstrom.** 2007. Semimechanistic pharmacokinetic/pharmacodynamic model for assessment of activity of antibacterial agents from time-kill curve experiments. Antimicrob. Agents Chemother. **51:**128–136.
22. **Nix, D. E., K. R. Matthias, and E. C. Ferguson.** 2004. Effect of ertapenem protein binding on killing of bacteria. Antimicrob. Agents Chemother. **48:** 3419–3424.
23. **Perry, T. R., and J. J. Schentag.** 2001. Clinical use of ceftriaxone: a pharmacokinetic-pharmacodynamic perspective on the impact of minimum inhibitory concentration and serum protein binding. Clin. Pharmacokinet. **40:** 685–694.
24. **Popick, A. C., W. G. Crouthamel, and I. Bekersky.** 1987. Plasma protein binding of ceftriaxone. Xenobiotica **17:**1139–1145.
25. **Riond, J. L., and J. E. Riviere.** 1989. Doxycycline binding to plasma albumin of several species. J. Vet. Pharmacol. Ther. **12:**253–260.
26. **Scaglione, F., G. Demartini, M. M. Arcidiacono, S. Dugnani, and F. Fraschini.** 1998. Influence of protein binding on the pharmacodynamics of ceftazidime or ceftriaxone against gram-positive and gram-negative bacteria in an in vitro infection model. J. Chemother. **10:**29–34.
27. **Schmidt, S., E. Schuck, V. Kumar, O. Burkhardt, and H. Derendorf.** 2007. Integration of pharmacokinetic/pharmacodynamic modeling and simulation in the development of new anti-infective agents—minimum inhibitory concentration versus time-kill curves. Expert Opin. Drug Discov. **2:**849–860.
28. **Schuck, E. L., and H. Derendorf.** 2005. Pharmacokinetic/pharmacodynamic evaluation of anti-infective agents. Expert Rev. Anti-Infect. Ther. **3:**361–373.
29. **Sevillano, D., M. J. Gimenez, L. Alou, L. Aguilar, F. Cafini, M. Torrico, N. Gonzalez, O. Echeverria, P. Coronel, and J. Prieto.** 2007. Effects of human albumin and serum on the in vitro bactericidal activity of cefditoren against penicillin-resistant Streptococcus pneumoniae. J. Antimicrob. Chemother. **60:**156–158.
30. **Stoeckel, K.** 1981. Pharmacokinetics of Rocephin, a highly active new cephalosporin with an exceptionally long biological half-life. Chemotherapy **27**(Suppl. 1)**:**42–46.
31. **Stoeckel, K., P. J. McNamara, R. Brandt, H. Plozza-Nottebrock, and W. H. Ziegler.** 1981. Effects of concentration-dependent plasma protein binding on ceftriaxone kinetics. Clin. Pharmacol. Ther. **29:**650–657.
32. **Suh, B., W. A. Craig, A. C. England, and R. L. Elliott.** 1981. Effect of free fatty acids on protein binding of antimicrobial agents. J. Infect. Dis. **143:** 609–616.
33. **Svensson, C. K., M. N. Woodruff, J. G. Baxter, and D. Lalka.** 1986. Free drug concentration monitoring in clinical practice. Rationale and current status. Clin. Pharmacokinet. **11:**450–469.
34. **Treyaprasert, W., S. Schmidt, K. H. Rand, U. Suvanakoot, and H. Derendorf.** 2007. Pharmacokinetic/pharmacodynamic modeling of in vitro activity of azithromycin against four different bacterial strains. Int. J. Antimicrob. Agents **29:**263–270.
35. **Van der Auwera, P., and J. Klastersky.** 1990. Study of the influence of protein binding on serum bactericidal titres and killing rates in volunteers receiving ceftazidime, cefotaxime and ceftriaxone. J. Hosp. Infect. **15**(Suppl. A)**:**23–34.
36. **Veronese, F. M., R. Bevilacqua, E. Boccu, and C. A. Benassi.** 1977. Drug-protein interaction: the binding of cephalosporins to albumins. Farmaco [Sci] **32:**303–310.
37. **White, R. L., M. B. Kays, T. A. Armstrong, and L. V. Friedrich.** 1990. Effects of supraphysiologic temperature and broth dilution on serum protein binding. Antimicrob. Agents Chemother. **34:**1257–1258.
38. **Yuk, J. H., C. H. Nightingale, and R. Quintiliani.** 1989. Clinical pharmacokinetics of ceftriaxone. Clin. Pharmacokinet. **17:**223–235.
39. **Zeitlinger, M., R. Sauermann, M. Fille, J. Hausdorfer, I. Leitner, and M. Muller.** 2008. Plasma protein binding of fluoroquinolones affects antimicrobial activity. J. Antimicrob. Chemother. **61:**561–567.
40. **Zeitlinger, M. A., R. Sauermann, F. Traunmuller, A. Georgopoulos, M. Muller, and C. Joukhadar.** 2004. Impact of plasma protein binding on antimicrobial activity using time-killing curves. J. Antimicrob. Chemother. **54:**876–880.