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The Impact of Protein Binding on Antibacterial Activities of Antibiotics is more than Predicted by Considering its Numerical Value Alone: Impact of Preparative and Incubation Methods on Different Pharmacodynamic Endpoints of ß-Lactams, Macrolides, Fluoroquinolones Against Gram-positive and Gram-negative Bacteria-Part I

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Abstract

Objectives: Protein binding decreases antibacterial activities as the free fraction only crosses membranes thus reaching intracellular targets. However, serum components may also increase antibacterial activities. Therefore, the effect of serum proteins on activities of ß-lactams, macrolides, and fluoroquinolones was examined. Several preparation- and cultivation-conditions were examined as some preparative methods like freeze-thawing of sera may cause artifacts. Furthermore, previous studies have indicated that data may vary depending on the endpoints studied. Therefore, the aim of this study was to avoid an impact of methodological factors on the data generated and to analyse the activities of the study drugs by examining different static- or dynamic endpoints.

Methods: Bacteria were grown in Brain Heart Infusion Broth (BHI), plus 50% of either fresh inactivated, pH 7.2, or fresh inactivated- , pH 7.2 or 8.2, frozen inactivated-serum, pH 8.2 as compared to BHI without any supplementations, pH 7.2 or 8.2, and BHI plus 45 g/L albumin. MICs of faropenem, amoxicillin, clarithromycin, azithromycin, moxifloxacin, and levofloxacin were determined and kill-kinetics were recorded following exposure to constant or fluctuating drug concentrations simulating serum pharmacokinetics of the agents. Kill-rates and areas under the bacterial kill curves were calculated.

Results: Albumin and inactive serum increased MICs and reduced kill-rates of the agents studied in conformity with their protein binding, whereas active serum increased the activities of the agents. MICs and kill-rates did not change in parallel. The impact of protein binding in decreasing order was: MICs>kill-rates in time-kill experiments>kill-rates in kinetic-simulations. Macrolides and fluoroquinolones but not ß-lactams were more active at an alkaline- than neutral pH. Use of frozen sera caused alkalinization of media, thus generating artifacts.

Conclusions: The impact of serum proteins on antibacterial activities is strongly dependent from three factors: the methods applied to prepare the serum pool, the incubation conditions, and the enpoints studied.

Keywords: Protein binding; Preparation; Storage; Alkalinization of serum; Artifacts; Discrete endpoints; Dynamic endpoints

Introduction

It is generally accepted that only the free fraction of antibiotics is antibacterially active. *In vitro* activities of most antibacterials decrease in parallel to increasing protein binding. Therapeutic *in vivo* efficacy of antibacterials, however, is the complex result of a number of factors. On the one hand, protein binding has a strong impact on minimal inhibitory concentrations (MICs) of antibacterials for the pathogen to be treated as well as on pharmacokinetics (PK) and pharmacodynamics (PD) of the agent as the free fraction only crosses pro- as well as eukaryotic membranes thus reaching intracellular targets or distributing between the intra- and extravascular spaces; furthermore, protein binding can alter renal excretion, half-life and volume of distribution thus affecting PK/PD-surrogates [1-5]. On the other hand, serum proteins protect the host from serious sequelae of an infection and/or may increase antibacterial activities. It was demonstrated that human immunoglobulin G [6,7] or unknown components present in human [8-20] and rat- or rabbit- serum [21,22] respectively, may increase antibacterial activities of a variety of drug classes.

A variety of physicochemical factors may have affected serum-antibiotic interactions. Storage of serum in the refrigerator rose the pH of the serum pool from 7.4 to ≥ 8.0 [23,24]. Changes in the pH of the test media have a significant effect on *in vitro* activities of macrolides, fluoroquinolones and aminoglycosides but leaving ß-lactams unaffected [25-29]. Furthermore, storage time and freeze-thaw cycles affect the stability of some serum proteins including albumin [30-32] and exposure of serum to heat as used for complement inactivation induced conformational changes of serum proteins affecting their interaction with bacterial membranes and antigens [33-39].

Some previously published data seem to be contradictory. It has been presented that MICs of cefotaxime, its metabolite

desacetylcefotaxime, or the combination of both remained unchanged in the presence of human serum while their bactericidal activities were improved [20]. The bacteriostatic activities of tetracyclines were converted into a rapid and pronounced bactericidal effect by fresh human serum [8]. Others described that bactericidal activities of highly protein bound dicloxacillin were reduced during the initial 6-hour phase of incubation only but not thereafter [40]. MICs of cefditoren, being bound to albumin to 88%, increased more than fourfold in the presence of albumin, but its bactericidal activity was reduced by albumin only and not by 90% human serum [41]; the authors concluded that the extrapolation of antibacterially active drug from the free fraction only results in an underestimation of antimicrobial activity [42,43]. Likewise, the bactericidal activity of tigecycline was underestimated if Gram-positive and Gram-negative indicator strains were exposed to the calculated free drug level whereas the agent was three-fold more active in the presence of albumin [44]. Although high protein binding of daptomycin (>90%) caused a three- to four-fold increase in MICs and MBCs [45], total daptomycin concentrations in the presence of human albumin or serum were bactericidally active against *E. faecium*, whereas the calculated free fraction was not [46]. Oritavancin retained bactericidal activity against enterococci in time kill assays although MICs increased up to eight-fold in the presence of albumin [47]. Serum proteins did not affect MICs of moderately protein bound ceftaroline or vancomycin whereas their bactericidal activities were significantly enhanced in the presence of active serum; bactericidal activities of highly protein bound ceftriaxone were augmented in the presence of active serum whereas its MICs increased more than four-fold [48]. These publications indicate that MIC- or MBC-determinations on the one hand and time-kill assays on the other hand generate discordant results. Discrete endpoint measures like MICs or MBCs and dynamic endpoints like time- and/or concentration- dependent reduction of viable counts evaluated in time kill assays have almost never been compared directly with each other, so that the question can be raised if data differ just because of methodological pitfalls or if the differences between discrete static- and dynamic endpoints, respectively, are real, and which of these endpoints may be representative.

Therefore, the aim of this study was to avoid as far as possible an impact of methodological factors on the data generated. Sera were collected in large batches and split into differently prepared fractions used in parallel for either discrete static- or dynamic endpoint determinations. Although differently protein bound agents were used it was not the aim of this study to investigate the impact of protein binding on their *in vitro* activities. Rather, the intention was to evaluate the effect of different preparation procedures like storage mediated pH shifts or conformational changes of serum proteins due to heat inactivation versus unprocessed serum (i.e. active serum) on the activities of the study drugs by examining different static- or dynamic endpoints.

Materials and Methods

Preparation of sera

Blood was sampled from twelve healthy volunteers and pooled; the volunteers did not take any tablets, in particular any antibacterials, since three months prior to sampling. Volunteers were almost on standard diet as they had breakfast and lunch in the same casino. Blood was allowed to clot at room temperature for 30 minutes and was centrifuged at 2,000 x g for 10 minutes. Serum was split into three parts; one part was used without any further processing (active serum) while the second part was heated at 56°C for 30 minutes (inactive serum) and used immediately thereafter (fresh inactivated serum); the third part was stored at -20°C, thawed and frozen daily for 7days and was used on the eighth day (frozen inactive serum).

Strains, antibacterial agents, and media

The following American Type Culture Collection (ATCC) wild type strains have been used throughout this study: *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 13597, and H*. influenzae* ATCC 33391. Strains have been adapted to growth in serum or body fluids in *in vivo* infection models and were maintained on blood agar plates. Brain Heart Infusion Broth (BHI; Becton Dickinson Diagnostics, Heidelberg, Germany), BHI + 50% each of heat inactivated human serum or active human serum, and 45 g/L human albumin (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) was used. The antibacterials were obtained from commercial sources. Antibacterial agents were dissolved and stock solutions were prepared according to manufacturer's instructions and stored in aliquots at -20°C. The antibiotic solutions were thawed and diluted in the appropriate media immediately prior to experiments. Heat inactivated- or active human serum as well as albumin was added to the antibiotic solutions after adjustment to room temperature. The final pH of the media was adjusted either to 7.2 or 8.2 immediately prior to use.

MIC testing and time-kill experiments

All flasks, microdilution trays, petri dishes etc. were siliconized to avoid adsorption of antibacterial agents studied to surfaces of glass- or plastic ware. MICs were determined according to CLSI guidelines [48]; time-kill curves were generated by exposure to multiples (1-, 4-, 8-, 16-, and 32-times) of the MICs recorded in the corresponding media to ensure that bioequivalent concentrations were used. Growth controls in the corresponding drug free media were run in parallel. Ciprofloxacin and *E. coli* ATCC 11775 served as controls. In general, MIC determinations or time-kill assays were run in parallel under the six experimental conditions studied and were repeated on a separate occasion; if data varied, the higher values are reported in the tables. Samples were withdrawn from the time-kill assays at 0, 1, 2, 4, 6, and 8 h after inoculation with 106 colony forming units (CFU)/mL. Viable counts were quantitated by plating serial dilutions of the sample in triplicate on BHI-agar plates; ß-lactams were inactivated by B. cereus ß-lactamase, macrolides and fluoroquinolones were inactivated by acidification of the medium. Drug concentrations in samples withdrawn at t=0h and t=8h were quantitated after centrifugation at 10,000g for 15 minutes by using the cup-plate diffusion assay with *B. subtilis* spore suspension as an indicator organism. Kill-rates (k, h-1) were calculated in analogy to growth-rates (μ, h-1) for the log-linear phase of declining CFUs [49]; kill rates were calculated for the drug exposed cultures whereas growth rates were calculated for the drug-free growth controls. As the maximal bactericidal activities of all the agents studied under any of the experimental conditions was achieved at eight times the MICs, data summarized in Table 1 represent the kill rates at this concentration.

Agent	Dose (mg)	PB (%)	Cmaxtotal(mg/L)	Cmaxfree(mg/L)	t_{max} (h)	$t_{1/2}$ (h)
Faropenem	150, b.i.d.	95	6	0.3	1.5, 13.5	0.9

Faropenem	300, b.i.d.	95	12	0.6	1.5, 13.5	0.9
Faropenem	600, b.i.d.	95	24	1.2	1.5, 13.5	0.9
Amoxicillin	250, b.i.d.	30	5.5	3.9	2.0, 14.0	1.2
Amoxicillin	500, b.i.d.	30	11	7.7	2.0, 14.0	1.2
Amoxicillin	875, b.i.d.	30	19.25	13.5	2.0, 14.0	1.2
Clarithromycin	500, b.i.d.	50	2.8	1.4	2.2, 14.2	3.5
Azithromycin	500, q.d.	15	0.2	0.2	2	2
Moxifloxacin	400, q.d.	40	3.3	2	3.3	13
Levofloxacin	500, q.d.	25	6.6	5	1	8
Levofloxacin	750, q.d.	25	8.6	6.5	1	8

PB: Protein Binding; Cmaxtotal: Maximal Total Serum Concentration; Cmaxfree: Maximal Calculated Free Serum Concentation; t_{max}: Time of Maximal Serum Concentration; $t_{½}$: Half Life, b.i.d: Twice Daily; q.d: Once Daily

Table 1: Pharmacokinetic parameters simulated in the Dialysis Tubing Model.

PK-simulations

Qualified calibrated and siliconized flasks were used for the PK-simulation studies. Serum pharmacokinetics were simulated by using a one compartment dialysis tubing model [50]; bacteria, serum or albumin were placed in a dialysis tubing (VISKING® Dialysis Tubing, molecular weight cut-off 12,000 – 14,000; pore diameter ca. 25 Å, Serva Electrophoresis GmbH, Heidelberg, Germany) to avoid wash out of bacteria or serum components from the central compartment and to reduce the amounts of serum needed in particular for the simulation of concentration-time profiles for antibiotics with short half-lives. The antibacterial agents studied diffused freely through the tubings. Tubings were boiled in sterile water containing 50 mM EDTA prior to use in the PK-simulation studies. Thereafter, bacteria +/- serum or albumin were placed into the tubings which were fixed within the central compartment. The system was allowed to equilibrate for 2 hours prior to addition of antibacterials. In general, samples were withdrawn from the interior of the tubings. CFUs were enumerated and drug concentrations were quantitated as described above.

The indicator strains were exposed to fluctuating drug concentrations as summarized in Table 1. Bacteria were exposed to total drug concentrations in BHI without any supplementation, BHI plus fresh inactive serum, BHI plus active serum, BHI plus 45 g/L albumin, as well as to the calculated free drug concentrations in BHI without any supplementation either at a pH of 7.2 or 8.2. Growth controls in the corresponding drug free media were run in parallel. The areas under the bacterial kill versus time curves (AUBKC, log10 CFU × h0-24 h/mL) were calculated using the trapezoidal rule [51,52].

The experiments were performed in four parts, i.e. 1st MIC-testing and time-kill experiments, 2nd PK-simulations, 3rd and 4th repetitions of these experiments on separate occasions, so that always the same group of volunteers donated blood on four occasions. Biochemical and hematological analysis of the four pooled serum samples revealed that all parameters were within the normal range on every occasion.

Results

Controls

All strains tested grew well throughout the entire incubation period under any of the experimental conditions studied. Growth rates were highly comparable under the different conditions studied as summarized in the companion publication [53]. The same holds true for PK-simulation studies as indicated by the AUBKC values of the drug-free controls ranging from 212.56 to 236.58 log10 CFU × h0-24 h/mL, individual values deviating from the mean AUBKC-value calculated for the drug-free controls by less than 5%. The activity of ciprofloxacin against *E. coli* ATCC 11775 used as an external control in the course of MIC- and time-kill assays was always within the limits of reproducibility. Drug concentrations quantitated prior to commencement and at the end of the time kill experiments corresponded to 96.7% to 99.4% to the weighed amount of substance in case total drug concentrations have been simulated. Monitoring of drug concentrations in the course of PK-simulation experiments revealed that within the tubings drug concentrations deviated from the desired profile on average by 4.6%, which is in agreement with previous studies. Interday and intraday reproducibility ranged from 93.2% to 97.8% as qualified calibrated flasks were used. No hysteresis within the dialysis tubings was noted.

Measurements of the pH values of frozen serum samples revealed that storage mediated shifts of pH values of frozen sera from initially 7.2 to 8.0-8.4 on day 8 occurred.

Impact of serum proteins on bacteriostatic and bactericidal activities

Albumin, fresh inactive serum at a pH of 7.2 (Table 2), and frozen inactive serum at a pH of 7.2 (data not shown) increased MICs and reduced kill rates of highly protein bound faropenem whereas moderately or minimally bound agents remained almost unaffected. Fresh active serum reduced the MICs of clarithromycin for *H. influenzae* as well as the MICs of azithromycin for the three indicator strains tested, whereas the MICs for the remaining drug-bug associations remained unaffected (Table 2). However, frozen inactive serum at a pH of 8.2 reduced the MICs of clarithromycin, azithromycin, moxifloxacin and levofloxacin, but not those of faropenem and amoxicillin. Analogous effects were caused by an alkalinization of BHI without any supplementations.

	Minimal inhibitory concentration (mg/L)	Kill rate (h^{-1})
	Brain Heart Infusion broth plus	Brain Heart Infusion broth plus

Agent/strain	nil, pH 7.2	nil pH 8.2	fresh inact ser pH 7.2	frozen inact ser pH 8.2	active serum pH 7.2	albumin pH 7.2	nil, pH 7.2	nil pH 8.2	fresh inact ser pH 7.2	frozen inact ser pH 8.2	active serum pH 7.2	albumin pH 7.2
Faropenem												
Sa 29213	0.12	0.12	0.25	0.25	0.12	0.25	-2.73	-2.71	-2.36	-2.35	-3.42	-2.29
Spn 13597	0.06	0.06	0.5	1	0.12	1	-1.66	-1.67	-1.42	-1.4	-2.42	-1.58
Hi 33391	0.25	n.g.	1	n.g.	0.5	2	-1.68	n.g.	-1.34	n.g.	-2.23	-1.13
Amoxicillin												
Sa 29213	0.12	0.12	0.12	0.12	0.12	0.12	-3.25	-3.24	-3.12	-3.12	-3.56	-3.18
Spn 13597	0.015	0.03	0.015	0.015	0.015	0.015	-1.48	-1.46	-1.46	-1.46	-2.68	-1.52
Hi 33391	0.25	n.g.	0.25	n.g.	0.12	0.25	-3.44	n.g.	.3.31	n.g.	.3.64	-3.24
Clarithromycin												
Sa 29213	0.12	0.03	0.5	0.03	0.25	0.5	-1.28	-1.57	-1.2	-1.37	-1.58	-1.2
Spn 13597	0.03	≤ 0.015	0.12	0.015	0.12	0.12	-0.5	-1.02	-0.42	-0.84	-2.05	-0.56
Hi 33391	16	n.g.	8	n.g.	4	16	-0.12	n.g.	-0.05	n.g.	-0.6*	-0.1
Azithromycin												
Sa 29213	1	0.25	1	0.25	0.12	1	-0.32	-0.67	-0.3	-0.62	-1.34	-0.3
Spn 13597	0.12	0.015	0.25	0.25	≤ 0.06	0.25	-1.63	-2.03	-1.59	-1.95	-3.62	-1.67
Hi 33391	1	n.g.	1	n.g.	0.25	1	-0.31	n.g.	-0.3	n.g.	-1.36	-0.29
Moxifloxacin												
Sa 29213	0.03	≤ 0.03	0.03	0.03	≤ 0.03	0.03	-4.33	-5.22	-4.31	-5.18	-5.33	-4.32
Spn 13597	0.12	≤ 0.03	0.12	0.12	0.06	0.03	-1.99	-2.87	-2.02	-2.94	-2.98	-2
Hi 33391	0.06	n.g.	0.06	n.g.	0.06	0.06	-2.07	n.g.	-2.1	-2.1	-2.69	-2.08
Levofloxacin												
Sa 29213	0.03	≤ 0.03	0.03	0.03	≤ 0.03	0.03	-4.12	-5.09	-4.15	-5.12	-5.13	-4.14
Spn 13597	0.12	≤ 0.03	0.12	0.12	0.06	0.12	-1.85	-2.77	-1.89	-2.85	-3.64	-1.85
Hi 33391	0.06	n.g.	0.03	n.g.	0.06	0.06	-1.99	n.g.	-1.95	n.g.	-2.74	-1.95
n.g: No Growth; *: Transiently bactericidal for 2 h to 3 h followed by regrowth in parallel to drug-free controls												

Table 2: Bacteriostatic activities expressed as minimal inhibitory concentrations (mg/L) and bactericidal activities evaluated in time kill experiments and expressed as kill rate (k, h) of *S. aureus* ATCC 29213 (Sa 29213); *S. pneumoniae* ATCC 13597 (Spn 13597), and H. influenzae ATCC 33391 (Hi 33391) incubated at the specified pH values either in Brain Heart Infusion Broth without any supplementation (nil), plus 50% freshly prepared heat inactivated serum (fresh inact ser), plus 50% frozen and thawed (8 cycles) heat inactivated serum (frozen inact ser), plus 50% freshly prepared active serum (active serum), or 45 g/L human serum albumin.

Kill rates of faropenem were affected by fresh or frozen inactive serum and albumin in parallel to modifications of MICs. However, active serum enhanced kill rates of faropenem despite its high protein binding. Active serum which did not cause any changes of MICs of amoxicillin or the fluoroquinolones increased their bactericidal activities as compared to protein free controls of fresh inactivated serum at a pH of 7.2. The augmentation of clarithromycin's and azithromycin's bactericidal activities by active serum at pH 7.2 was more distinct than the effect on kill rates caused by an alkalinization of the medium (Table 2).

PK-simulation studies

Frozen inactivated serum adjusted to a pH of 8.2 has not been studied in this series of experiments as the serum pool was limited. In general, the activities of calculated free drug concentrations in BHI without any supplementations on the one hand and the activities of study drugs in the presence of inactive serum or albumin on the other hand differed by 1% to 16%. Alkalinization of media increased the activities of fluctuating concentrations of clarithromycin, azithromycin, moxifloxacin and levofloxacin, but not the activities of faropenem or amoxicillin (Table 3). Active serum increased the activities of sub-optimal dosing regimens more clearly than the activities of adequate regimens. Twice daily doses of 150 g faropenem or 250 mg amoxicillin, for example, reduced viable counts of *H. influenzae* or *S. pneumoniae* grown in media supplemented with fresh inactivated serum or albumin minimally whereas viable counts of these two indicator strains were reduced rapidly and effectively in the presence of active serum (Table 3). The activities of both agents were much higher in the presence of active serum than their activities in unsupplemented broth. The same holds true for clarithromycin and azithromycin. A similar trend, although less marked, was seen for moxifloxacin and levofloxacin.

	Brain Heart Infusion Broth plus											
Agent/strain	Nil, total		Nil, calculated free, pH 7.2		Nil, calculated free, pH 8.2		Inactive serum		Active serum		Albumin	
	pH 7.2	pH 7.2					pH 7.2		pH 7.2			
	AUBKC	k	AUBKC	k	AUBKC	k	AUBKC	k	AUBKC	k	AUBKC	k
FAR 150 mg												
Sa 29213	58.6	-3.27	68. 97	-3. 55	68.2	-3.49	59.85	-2.87	19.45	-3.77	63.3	-3
Spn 13597	64.73	-3.25	206.78	-0.03	207.02	0	192.45	-0.33	15.4	-6	188	-0.46
Hi 33391	184.55	-0.74	199.35	0.08	n.g	n.g.	195.72	-0.14	17.85	-4.67	195.77	-0.13
FAR 300 mg												
Sa 292913	21.75	-3.46	35.89	-2.78	n.d	n.d	27.75	-3.06	13.45	-4.2	26.45	-3.27
Spn 13597	55.09	-3.31	209.37	-0.02	n.d	n.d	193.45	-0.4	12.35	-6.44	194.8	-0.53
Hi 33391	189.65	-1.87	209.68	0.12	n.g	n.g	202.3	-0.2	14.55	-7.22	207.65	0.09
FAR 600 mg												
Sa 29213	16.6	-3.8	20.02	-2.59	n.d	n.d	21.45	-3.06	13.45	-4.2	16.45	-3.27
Spn 13597	46.9	-3.73	182.26	-0.66	n.d	n.d	177.55	-0.53	10.5	-6.67	179.65	-0.73
Hi 33391	171.25	-1.93	202.79	-0.11	n.d	n.d	201.11	-0.34	13.85	-7.45	204.05	0.13
AMX250mg												
Sa 29213	44.1	-2.77	41.35	-2.88	41.55	-2.82	47.35	-2.53	14.1	-3.54	44.15	-2.67
Spn 13597	101.6	-2.87	100.23	-2.69	99.89	-2.59	114.5	-2.55	15.85	-5.67	115.7	-2.74
Hi 33391	162.4	-1.67	158.45	-1.62	n.g.	n.g	168.2	-1.6	34.85	-4.06	168.15	-1.54
AMX500mg												
Sa 29213	21.75	-3.47	19.84	-3.28	n.d.	n.d	25.6	-3.2	15.5	-4.67	22.35	-3.27
Spn 13597	100.35	-2.94	102.23	-2.88	n.d.	n.d	110.9	-2.8	9.6	-6.78	117.5	-2.67
Hi 33391	149.65	-1.94	145.56	-1.92	n.d.	n.d	153.7	-1.87	26.85	-4.06	153	-1.87
AMX875mg												
Sa 29213	20.95	-3.54	19.56	-3.48	n.d.	n.d	21.3	-3.4	14.55	-4.89	21.5	-3.4
Spn 13597	99.1	-3	97.67	-2.85	n.d.	n.d	103.5	-2.87	10.25	-6.78	99.95	-3
Hi 33391	149.6	-1.87	141.26	-1.85	n.d.	n.d	149.55	-2	26.2	-4.14	152.4	-1.93
CLR 500mg												

Sa 29213	127.6	0.28	202.41	0.36	96.82	-0.19	195.92	0.32	59.73	-1.09	200.53	0.34
Spn 13597	118.88	-0.26	172.85	-0.14	92.41	-0.79	168.24	-0.19	42.55	-0.46	171.14	-0.15
Hi 33391	n.d.	n.d	n.d.	n.d	n.g.	n.g	n.d.	n.d	n.d.	n.d	n.d.	n.d
AZM500mg												
Sa 29213	172.97	0.42	170.32	0.39	159.28	-0.08	171.02	0.37	33.85	-1.22	172.22	0.4
Spn 13597	176.96	0.39	173.86	0.36	169.82	-0.13	179.25	0.43	28.92	-3.38	175.88	0.37
Hi 33391	202.83	0.56	206.34	0.54	n.g.	n.g.	204.55	0.55	84.1	-1.42	200.97	0.54
MXF400mg												
Sa 29213	82.55	-2.56	83.59	-2.49	67.87	-2.96	82.69	-2.39	55.73	-2.98	83.59	-2.49
Spn 13597	69.93	-3.72	71.89	-3.69	59.66	-4	69.89	-3.69	37.89	-4.55	71.89	-3.69
Hi 33391	67.85	-1.78	69.37	-1,74	n.g.	n.g	71.17	-1,74	25.37	-2.99	69.37	-1,74
LVX 500mg												
Sa 29213	76.55	-1.82	76.28	-1.77	70.16	-2.25	77.28	-1.67	62.63	-2.97	76.28	-1.77
Spn 13597	74.66	-2.24	75.28	-2.21	68.56	-2.89	76.18	-2.11	59.73	-3.26	75.28	-2.21
Hi 33391	63.46	-1.98	64.39	-1.93	n.g.	n.g.	66.22	-1.84	45.67	-2.32	64.39	-1.93
LVX750mg												
Sa 29213	69.55	-2.03	69.98	-2	61.13	-2.68	69.98	-1.89	53.46	-3.24	69.98	-2
Spn 13597	61.43	-2.79	63.21	-2.74	47.53	-3.1	63.21	-2.68	41.39	-3.97	63.21	-2.74
Hi 33391	59.77	-2.56	60.59	-2.51	n.g.	n.g	60.59	-2.43	42.37	-2.89	60.59	-2.51
n.g: No Growth; n.d: Not Done												

Table 3: Antibacterial activities (expressed as area under the bacterial kill curve (AUBKC, log10 CFU × h/mL) and as kill-constant (k in h; the kill constants are expressed as negative figures, a + indicates growth) of simulated faropenem (FAR)- and amoxicillin- (AMX) serum-concentrations. Growth controls in drug free medium were run in parallel; the three indiator strains grew equally well in Brain Heart Infusion Broth supplemented with active- or inactive serum or human albumin with AUBKC-values ranging from 212.56 to 236.58 log10 × h/mL.

Discussion

Data generated in this study demonstrate that the methods applied to prepare and to store sera as well as the endpoints evaluated have a significant impact on the data generated and the conclusions to be drawn. The results confirm that only the free fraction of antibacterial agents was active against the indicator strains tested, provided the bacteria were incubated at a neutral pH in inactivated serum or albumin. MICs of the agents increased and kill rates decreased in conformity with their protein binding. Likewise, PK-simulation experiments revealed that highly protein bound faropenem was less active in the presence of inactive serum or albumin, whereas the activity of moderately protein bound amoxicillin was minimally affected. Also, the activities of calculated free drug concentrations were within the same range as the activities of the study drugs in the presence of inactivated serum or albumin. The calculation of free drug concentrations based on published data was significantly detracted by highly variable data. For example, protein binding of clarithromycin may be as low as of 42 to 50% over a concentration range of 0.25 to 0.5 mg/L [54], or as high as 72% over a concentration range of 0.04 to 1.0 mg/L [55,56]. Protein binding of moxifloxacin being concentration independent was found to range from 25% [57] to 52% [58]. Such differences really matter, as the free fraction used as basis for PK/PD calculations could be assumed to be as low as 30% and 48% or as high as 60% and 75%, respectively. These differences are very likely due to methodological differences as reviewed previously [3,59], but the question is which of the published data should be selected for PK/PD calculations and based on which criteria?

Another open question is which preparative method should be used. Unprocessed serum has been used without any storage in this and other studies [7,8,12,13,15], while others [10,11,17,18] used serum-ultrafiltrate being devoid of peptides. Serum-antibiotic synergisms were obtained with sera prepared by either method, so that not only serum proteins, but also low molecular weight unknown factors may have contributed to the synergistic effects. Furthermore, the pH of the medium used had a significant impact on the data generated in this study. Alkalinization of unsupplemented BHI caused an increase in antibacterial activities of clarithromycin, azithromycin, moxifloxacin and levofloxacin, whereas the activities of faropenem and amoxiclilin remained unaffected. Likewise, a storage mediated shift in pH values of frozen sera from 7.2 to 8.0-8.4 caused a decrease in MICs and an increase in kill-rates, whereas this effect was not noted in frozen sera adjusted to a pH of 7.2 or in fresh and unprocessed sera.

Thus, this effect is clearly due to an alkalinization of serum preparations mediated by freeze-thaw cycles. It has been described previously that addition of serum caused an increase in potency of azithromycin due to an increase in pH [60]. These findings are very well in agreement with the physicochemical characteristics of the agents studied. Macrolides, azithromycin in particular, and fluoroquinolones exist in their least ionized- and thus antibacterially most active state at an alkaline pH, so that the storage mediated alkalinization of the serum preparation favours the activities of these drug classes, whereas the amphoteric ß-lactams amoxicillin and faropenem are least ionized at an acidic pH, so that these agents were not affected by such storage mediated pH shifts. The impact of storage mediated pH shifts on antibacterial activities of antibiotics in the presence of serum passed unnoticed in the past, as the pioneering studies performed by Rolinson et al. have been performed with amphoteric ß-lactams [23,61-64]. Data for macrolides and fluoroquinolones generated in this study demonstrate that the routine procedure to store serum samples at -20°C may very likely to cause artifacts in case the pH values of the preparations are not adjusted – which is at least not reported in the relevant publications. Not only the pH of the medium, but also the use of either inactivated or active serum has a significant impact on MICs as well as kill rates as active serum augmented the activities of the agents studied; this phenomenon will be discussed in the companion manuscript [53]. Thus, not only the methods to quantitate serum protein binding, but also preparative- and storage as well as incubation- methods have to be standardized, as exact determinations of free drug concentrations and their MICs are the cornerstones for PK/PD calculations. But – which MIC value should be used? The one determined by exposure to total drug concentrations in the absence of serum proteins, as done routinely right now, or those MICs determined in the presence of either inactivated or active serum proteins? MICs quantitated in the presence of fresh active serum are probably clinically most relevant. Furthermore, it is reported in the companion manuscript that disproportionate increases in MICs of faropenem (albumin bound) and roxithromycin (α-1-acidic glycoprotein bound) were observed in albumin- and serum- supplemented BHI, respectively, although both agents are highly protein bound, so that probably the differential effect of different serum protein factors should be considered.

A comparison of different endpoints evaluated in the course of this study revealed that the impact of serum proteins on the discrete static endpoint MIC as compared to the dynamic endpoint kill rate was different. Although the MICs of faropenem or clarithromycin increased two- to fourfold in the presence of inactivated serum or albumin at a pH of 7.2, the kill rates following exposure to constant- or fluctuating drug concentrations did not decrease in parallel, if at all; on the contrary, kill rates were augmented in the presence of active serum. Kill rates of amoxicillin were much higher in active serum, too, despite its low protein binding. This disconnect between MICs and kill rates is very well in agreement with previously published data [8,20,40-47]. Wouldn't it be more conclusive to derive PD measures not from static but from dynamic endpoints like kill-rates [65]? In summary, it can be concluded that diverse methods affect discrete static or dynamic endpoints differently.

Already in 1942 Davis studied the impact of protein binding properties of sulfonamide on pharmacokinetics and clinical outcome and concluded that "it is probable that only unbound drug is bacteriostatically active" [66,67]. Despite almost 75 years of research on protein binding of antibacterials no methodological or performance standards for such investigations has been developed. Standards should be developed in analogy to MIC testing based on interlaboratory studies.

Limitations

The impact of preparative methods and incubation conditions on bacteriostatic or bactericidal antibacterial activities of different drug classes has been studied. Although it was not the aim of this study to investigate the impact of protein binding as such on antibacterial activities of the agents studied it would have been interesting to evaluate if various preparative methods may have affected the association- and dissociation-constants as well as the overall binding capacities. It has been demonstrated that e.g. binding of erythromycin to human serum proteins is affected by drug concentration, temperature and pH [54,68-70]. It would therefore have been desirable to quantitate protein binding as well as free drug concentrations not only in the different serum preparations studied but also in the course of PK-simulations because of continuously fluctuating drug concentrations. However, quantitation of free drug concentrations is laborious and time consuming depending on the methods applied [3,59], so that total drug concentrations in the 7,224 samples withdrawn in this study were assayed instead and unspecific adsorption was avoided by siliconization of the glass- and plastic ware used. Although total drug concentrations varied by <5% from the theoretical concentrations suggesting that methodological errors can be excluded, this finding does not indicate that free drug levels were identical under the various conditions studied. Furthermore, data generated demonstrate that protein binding of the agents studied affected their bacteriostatic- or bactericidal activities differently. Every effort has been made to apply identical test conditions, so that the differences between discrete static- and dynamic endpoints, respectively, are not due to varying performance criteria. But an explanation why diverse methods affected discrete static or dynamic endpoints differently cannot be provided. Further studies would be desirable to address this open question.

Conclusion

The impact of serum proteins on antibacterial activities of macrolides, fluoroquinolones, and ß-lactams against the strains studied is strongly dependent from three factors: the methods applied to prepare the serum pool, the incubation conditions, and the endpoints studied.

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Author's Contributions

A.D. and S.S. have contributed equally to the design and execution of the work described as well as to the analysis and interpretation of the data generated and have drafted and revised the manuscript.

Transparency declaration

None to declare

Ethics

Not applicable

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