Experimental Pitfalls in the Measurement of Protein Binding

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Introduction

Antibiotic resistance has become a continuously increasing problem, especially in treatment of nosocomial infections [1]. In order to optimize antibiotic treatment, the primary goal has to be to evaluate possible influence factors that contribute to resistance.

Numerous in vitro experiments have shown that Protein Binding (PB) is an important factor for antimicrobial activity, especially for highly bound antibiotics. However, the experimental conditions that best simulate the *in vivo* situation are still subject of controversy.

Objectives

Therefore, the aim of this study was to determine the effect of protein binding PB on the antimicrobial activity of highly bound ceftriaxone (PB 95-98%) and evaluate the potential influence of culture media and/or albumin supplementation vs. plasma in three comparative, dose-ranging *in vitro* experiments:

- Determination of free, unbound ceftriaxone, performing microdialysis experiments in different (growth) media
- Linkage of free, unbound ceftriaxone concentrations to its antimicrobial activity, performing minimum inhibitory concentration (MIC) and time-kill curve determinations
- Evaluation of potential differences in the effect of PB between gram-positive and gram-negative bacteria

To set up a PK/PD model that allows to predict the antimicrobial activity of ceftriaxone at any concentration against the pathogen used.

Methods

Analytics

•Determination of free, unbound concentration

A comparative, dose-ranging in vitro microdialysis study was conducted to determine free, unbound ceftriaxone concentrations in Lactated Ringer's solution and Todd Hewitt Broth (THB) both with and without bovine serum albumin (BSA; Sigma, St. Louis) 40g/L and human plasma at 37°C. Sample were analyzed, using a modified RP-HPLC 18 method.

•Determination of MICs/time-kill curves

Using a modified broth dilution method, the MICs were determined *in vitro* both in presence and absence of bovine serum (BSA) albumin 40g/L (bovine serum, fraction V, approx. 99%, SIGMA-ALDRICH, Inc.) in Mueller-Hinton broth (MHB, Becton Dickinson) and Todd Hewitt broth (THB, Difco). Gram-negative Escherichia coli (E. coli) ATCC 25922 and gram-positive, penicillin-sensible Streptococcus pneumoniae (S. pneum.) ATCC 6303 were used as the test organisms.
E. Coli ATCC 25922 and S. pneum. ATCC 6303 were equilibrated *in vitro* for two hours in MHB in vented cap culture tissue flasks to ideally reach the log growth phase. After two hours, cultures with 7 different concentrations of ceftriaxone, ranging from 0.25xMIC to 16xMIC, plus two growth controls (with and without albumin) were used to set up the time-kill curve. Samples were taken at time points 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6 hours and the change in number of bacteria (CFU/mL) versus time was linked to its effect.

Simulation

A model was set up which fits the data well. The data was modeled using the software Scientist® for Windows™ (MicroMath®, Salt Lake City, Utah) and the following modified sigmoid Emax model was used.

$$\frac{dN}{dt} = \left(k_0 \cdot \left(1 - \frac{N}{N_{max}}\right) \cdot \left(1 - e^{-\beta t}\right) - \frac{k_{max} \cdot C^h}{EC_{50}^h + C^h} \cdot \left(1 - e^{-\alpha t}\right) \right) \cdot N$$

Applied model: *N = starting number of bacteria in the flask, N_{max} = maximum number of bacteria in the flask, C = concentration of ceftriaxone in the culture flask, α = delay in kill, β = delay in growth, , T = time k_0 = growth rate constant, k_{max} = maximum kill rate constant, EC_{50} = concentration at 50% of maximum effect, h = hill/shape factor*

Results

In vitro microdialysis

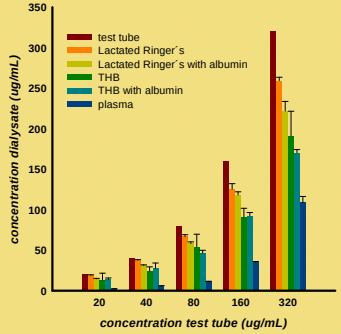
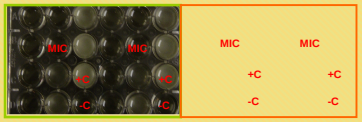


Figure 1: *measured unbound ceftriaxone concentrations*

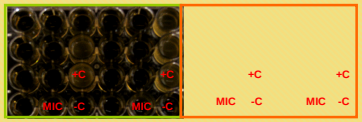
Statistical analysis was performed for every single concentration (20, 40, 80, 160, 320µg/mL), using GraphPad PRISM® and employing an one-way ANOVA test, followed by a Tukey test (post test) with a confidence interval of 95% (P<0.05).

- no statistically significant differences between Lactated Ringer´s with vs. without albumin (P>0.05)
- no statistically significant differences between THB with vs. without albumin (P>0.05)
- free concentrations in THB, (with and without albumin were significantly lower compared to Lactated Ringer´s (P<0.05)
- compared to plasma all differences in free concentration were highly significant (P<0.001) apart from THB with albumin (P<0.01)

MICs



Picture 1: *ceftriaxone against E. coli ATCC 25922 without and with bovine albumin 40g/L;*



Picture 2: *ceftriaxone against S. pneum. ATCC 6303 without and with bovine albumin 40g/L;*

Time-kill curves

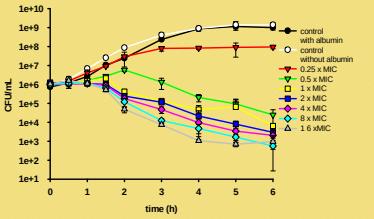


Figure 2: *Mean data curve of ceftriaxone vs. E. coli ATCC 25922 without bovine albumin, number of repetitions N=4*

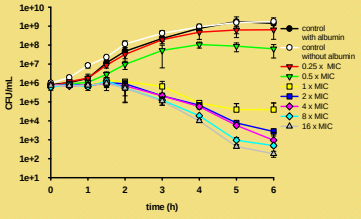


Figure 3: *Mean data curve of ceftriaxone vs. E. coli ATCC 25922 with bovine albumin 4g/L, number of repetitions N=4*

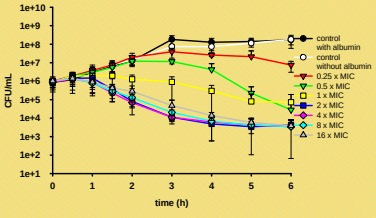


Figure 4: *Mean data curve of ceftriaxone vs. S. pneum. ATCC 6303 without bovine albumin 40g/L, number of repetitions N=3*

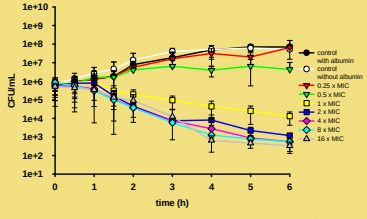
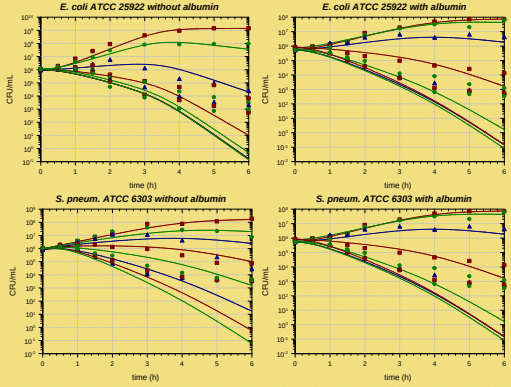


Figure 5: *Mean data curve of ceftriaxone vs. S. pneum. ATCC 6303 with bovine albumin 40g/L, number of repetitions N=3*

Simulations



parameter [unit]	E. coli ATCC 25922		S. pneum. ATCC 6303	
	w/out	*w/*	*w/out*	*w/*
MIC [µg/mL][1]	0.064	0.064	0.010	0.020
k_0 [h^{-1}]	2.52	2.25	1.30	1.46
kmax [h^{-1}]	4.31	4.11	4.55	3.816
α [h^{-1}]	1.59	0.67	1.55	1.65
β [h^{-1}]	1.90	1.87	17.54	1.91
EC_{50} [µg/mL]	0.033	0.046	0.022	0.019
h	2.25	4.32	1.08	2.42

Table 1: *determined/modeled parameters*

Discussion and Conclusions

In the first place this study surprisingly showed that protein binding did not have the effect on the MIC of the highly bound beta-lactam ceftriaxone that one might have expected. However, having a closer look, this was not due to the make-up of the MIC experiment itself but, as shown in the *in vitro* microdialysis experiment, a relatively higher free, antimicrobial active amount of drug. Determination of the actual protein binding then showed a statistically significant lower degree of PB of BSA compared to pooled human plasma (P<0.05). These findings were confirmed in additional, more reliable time-kill curve experiments. Fitting the data to a modified E_{max} model did not result in statistically significant differences (P<0.05) in determining k_{max} and EC_{50} values. This held true for both gram-positive Streptococci pneumoniae and gram-negative Escherichia coli tested.

In conclusion, this study clearly showed that 1) free, unbound drug is responsible for efficacy and 2) one has to be very careful with making assumptions about the degree of PB based on literature values. Instead, the actual PB has to be measured in the system used.

References

1. Nightingale, C., Murakawa T., Ambrose, P. (2002). Antimicrobial Pharmacodynamics in Theory and Clinical Practice, Marcel Dekker, Inc., 2-9
2. Derendorf, H., Schuck E. L., Liu, Q., Khunvichai, A. (2004). MIC- VS. KILL CURVE-BASED PK/PD INDICES. Poster ICAAC 2004, Department of Pharmaceutics, University of Florida, Gainesville, FL.
3. Eagle, H., Fleischmann, R., Musselman, A. D. (1950). Effective concentrations of penicillin in vitro and in vivo for streptococci, pneumococci, and Treponema pallidum. *J. Bacteriol.* 59, 625-43
4. Leggett, J. E., Craig, W. A. (1989). Enhancing effect of serum ultrafiltrate on the activity of cephalosporins against gram-negative bacilli, *Antimicrob. Agents Chemother.* 33, 35-40