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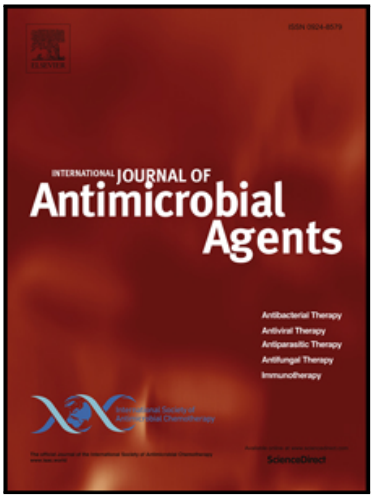

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Interspecies differences in Plasma Protein Binding of Beta-Lactam Antibiotics

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Abstract

Background

Plasma protein binding (PPB) is a critical factor in drug therapy and understanding free compound exposure across preclinical and clinical species is vital for developing new antibiotics. Optimizing beta-lactam dosing based on unbound drug concentrations has garnered significant interest, yet comprehensive data on how inter-species differences in protein binding affect the attainment of targeted unbound concentrations remain sparse.

Methods

This study aimed to examine the protein binding of three beta-lactams: cefiderocol, ceftriaxone, and temocillin using human, bovine, and rat plasma. Total and unbound beta-lactam concentrations were measured through ultrafiltration. An interspecies comparison of PPB was conducted to evaluate variability in protein binding across the different species.

Results

The findings revealed that PPB was highest in human plasma for all three beta-lactam antibiotics tested. In rat plasma, PPB was higher for cefiderocol and ceftriaxone compared to bovine plasma, while bovine plasma exhibited higher PPB for temocillin compared to rat plasma.

Conclusion

Significant variability in protein binding was observed among and between different species for the tested drugs. The study highlights substantial interspecies differences in the plasma protein binding of cefiderocol, ceftriaxone, and temocillin. Our findings indicate the need for careful consideration of species-specific PPB in the optimization of beta-lactam dosing and the development of new pharmaceuticals.

Keywords: Plasma protein binding, interspecies differences, beta lactam, cefiderocol, ceftriaxone, temocillin, ultrafiltration.

Introduction

The pharmacokinetic (PK) properties of antibiotics play a pivotal role in determining their efficacy and safety profiles [1]. Among these properties, plasma protein binding (PPB) stands out as a crucial parameter influencing drug distribution, clearance, and ultimately, therapeutic outcomes, since only the unbound fraction of an antibiotic is considered to be antimicrobially active [2]. Understanding the PPB of antibiotics, especially in comparison across species, is essential for translational research and clinical practice, facilitating the extrapolation of findings from preclinical models to humans [3].

In our prior study, the primary focus was on understanding the interspecies PK/PD of cefazolin, a first-generation beta-lactam antibiotic widely used against various bacterial infections. The study encompassed investigations of the drug's PPB, its susceptibility in vitro to *Escherichia coli*, bacterial growth across different media, and time-kill curves under diverse plasma conditions [3]. Given the significance of this research area, our current study expanded investigation of interspecies PPB profiles to three important antibiotics: cefiderocol, ceftriaxone, and temocillin. Cefiderocol, a novel siderophore cephalosporin, demonstrates promising activity against multidrug-resistant Gram-negative pathogens [4]. The unique features of cefiderocol as well as its promising *in vitro* and *in vivo* results suggest makes it a potentially valuable addition among the limited therapeutic options available in the treatment of multi/drug resistance infections [4]. Ceftriaxone, a third-generation cephalosporin, has been a mainstay in clinical practice for decades due to its broad spectrum of activity and favourable pharmacokinetic properties [5]. Temocillin, a beta-lactam antibiotic, holds potential as a therapeutic option for infections caused by multidrug-resistant Gram-positive bacteria [6]. With its notable attributes including an extended half-life of approximately 4 hours, and satisfactory distribution and efficacy in extracellular spaces, temocillin emerges as a promising therapeutic option [7]. However, its considerable and self-saturating PPB at clinically relevant concentrations presents challenges in optimizing dosing strategies based on pharmacokinetic/pharmacodynamic (PK/PD) principles.

Equilibrium dialysis is widely recognized as the gold standard for determining the PPB *in vitro* [8]. However, ultrafiltration has become increasingly popular due to its much shorter processing time [8]. Therefore, we employed this technique to characterize the PPB profiles of these antibiotics across multiple species, including human, bovine, and rat. Such comparative analyses provide valuable insights into interspecies differences in drug binding to plasma proteins.

Materials and methods

Antibiotics and plasma

Cefiderocol, ceftriaxone, and temocillin were purchased from Sigma-Aldrich and prepared/preserved throughout the experiments in accordance with the manufacturers' instructions. Human plasma was purchased from Octapharm, while rat plasma was obtained from Sigma-Aldrich (Germany). Bovine plasma was supplied by the Veterinary department of the Medical University of Vienna.

Quantification of PPB of cefiderocol, ceftriaxone, and temocillin through ultrafiltration (UF)

We conducted UF as described before [3] to elucidate the PPB of cefiderocol, ceftriaxone, and temocillin in human, rat, and bovine plasmas. Our experiments utilized 20%, 70%, and 100% concentrations (diluted with PBS) of human, rat, and bovine plasmas for PPB assessments. In drug development, generally rodent species and non-human primates are used. We also included bovine plasma in our study as bovine plasma shares several biochemical properties with human plasma, including comparable albumin concentrations, buffering capacity, and enzymatic activity. Moreover, bovine plasma provides a broader interspecies comparison of plasma protein binding (PPB) for the beta-lactam antibiotics. Its inclusion allowed the investigation of PPB variability across a non-traditional species, complementing data from human and rat plasma. Drugs' concentrations were chosen to mirror typical serum levels following standard human doses [9,10,7].

In our study, three drugs—cefiderocol, ceftriaxone, and temocillin—were evaluated at multiple concentrations to capture a range of pharmacological conditions:

Cefiderocol: 5 mg/L, 25 mg/L, and 200 mg/L

Ceftriaxone: 10 mg/L, 80 mg/L, and 400 mg/L

Temocillin: 7.5 mg/L, 50 mg/L, and 250 mg/L

These concentrations were chosen to span the spectrum of therapeutic and supra-therapeutic levels, ensuring the inclusion of clinically relevant exposures. Observed steady-state plasma concentrations in humans at standard dosing for cefiderocol [11], ceftriaxone [12] and temocillin [7] were used as a reference.

Plasma samples from human, rat, and bovine sources were stored at -20°C, thawed, and centrifuged at approximately 460 RCF for 5 minutes to remove any precipitates. Subsequently, the pH was adjusted to 7.4 by adding NaH_2PO_4. A 1 mL sample of each drug-spiked plasma was prepared by directly diluting the drug stock solution into the plasma. The spiked samples were incubated at 37°C for 30 minutes. Stability of the drugs have been tested previously, and available evidence suggests that all three drugs exhibit stability under these conditions [13–15]. Subsequently, 250 µL aliquots of each combination were dispensed into a centrifugal filter unit containing a low-binding regenerated cellulose membrane (Ultrafree-MC™, nominal molecular weight limit 5000; Millipore Corp., USA). The ultrafiltration process was carried out through centrifugation at 1410 RCF for 40 minutes at room temperature. Each UF procedure was conducted in triplicate. PPB was calculated as: PPB (%) = 100% - $C_{UF}/C_{Serum} \times 100\%$, with C_{UF} being the (free) drug concentration in the ultrafiltrate, and C_{Serum} the total drug concentration in the retained serum sample.

HPLC Analysis

The determination of drugs' concentrations in human, bovine, and rat plasma samples, as well as in ultrafiltrate, was conducted through high-performance liquid chromatography (HPLC). Chromatography was performed on a Prominence Modular LC-20 series consisting of degasser DGU 20A3R, quaternary solvent pump LC 20AD with low pressure gradient mixer, autosampler SIL 20AC HT (set at 6°C), column oven CTO 20AC (set at 40°C), photodiode array detector SPD M30A, equipped with cells of 10 mm or 85 mm optical path length, system controller CBM 20A and LabSolution soft-ware (Shimadzu, Duisburg, Germany). The detection wavelength was 245 nm (temocillin), 260 nm (cefiderocol) or 285 nm (ceftriaxone).

Total drug concentrations were analysed following a repeatedly published protocol, e.g. [16]. In brief, serum (100 µL) was buffered with 20 mM sodium phosphate buffer, pH 6.0, (200 µL) and deproteinized with acetonitrile (500 µL). The precipitated protein was separated by centrifugation, the acetonitrile was extracted into dichloromethane (1.5 mL) and 1 µL aliquot of the aqueous layer was injected. Ultrafiltrate was injected directly. Separation was performed on a Cortecs T3 2.7 µm column (i.d. 100x3 mm, Waters, Eschborn, Germany). The mobile phase for the analysis of cefiderocol and ceftriaxone was 0.1 M sodium phosphate buffer/acetonitrile, pH 3.0, 88:12 (v/v). Ceftriaxone eluted after 2.9 min, cefiderocol after 3.5 min (flow rate 0.4 mL/min). The mobile phase for the analysis of temocillin was 100 mM sodium ammonium acetate/acetonitrile 97:7 (v/v), pH 6.7. Temocillin eluted after 4.4 min (R-isomer) and 5.3 min (S-isomer), respectively. The calibration standards were prepared in human serum (analysis of the retained serum) or in saline (analysis of ultrafiltrate). The linearity of the assays (cefiderocol, ceftriaxone, sum of R/S-temocillin) has been proven down to 1 mg/L. The imprecision based on QC samples in serum (for the analysis of the retained serum) or saline (for the analysis of ultrafiltrate) was <5% and <3%, respectively. The accuracy in serum was between 99.5% and 105% and in saline between 97.3% and 102%, respectively.

Further validation is summarized in Supplementary Table S1.

Statistical calculations

Statistical analyses were conducted using commercially available software (GraphPad Prism, San Diego, California USA, www.graphpad.com). Paired t-test analysis of the means of PPB data was performed among human, bovine and rat plasmas. A P value <0.05 was considered statistically significant.

Results

Protein binding of cefiderocol, ceftriaxone, and temocillin

Mean protein binding (± standard deviation) values for cefiderocol, ceftriaxone, and temocillin using UF are depicted in Figure 1A, 1B and 1C, respectively. Cefiderocol had low protein binding in all media as compared to the other tested antibiotics in this study, which increased with an increase in the concentration of plasma and was highest in 100% plasma (Figure 1A). PPB was significantly higher in humans as compared to that in bovine (p-value=0.0002) and rat (p-value=0.0003). Bovine displayed less PPB as compared to that in human and rat and this tend becomes more pronounced in 100% plasma. Moreover, rat showed similar protein binding to human at lower drug concentration (5µg/ml) but PPB of rat decreases as compared to human at higher drug concentrations (25 and 200 µg/ml). Paired t-test analysis of the means from the rat PPB data at three different concentrations of cefiderocol (200, 25, and 5 µg/ml) indicated that they were significantly higher to that observed for the bovine data at all tested plasma concentrations (p-value < 0.0002).

Ceftriaxone displayed high protein binding among the tested drugs in human and rat plasma that increased with an increase in the concentration of plasma and was highest in 100% plasma (Figure 1B). PPB was significantly higher in humans as compared to that in bovine (p-value < 0.00006) and rat (p-value=0.00007). Bovine PPB was lowest as compared to that in human and rat. Besides, highest bovine PPB was found to be in 100% plasma. Moreover, rat had lower PPB as compared to human and this pattern more evident at highest drug concentration (400 µg/ml). Paired t-test analysis reveals that PPB of ceftriaxone in rat was found to be significantly higher as compared to bovine (p-value < 0.0004) at the investigated plasma concentrations (20%, 70%, and 100%).

Temocillin displayed low protein binding at 20% plasma concentration that increased with an increase in the concentration of plasma and was highest in 100% plasma (Figure 1A). PPB was highest in humans as compared to that in bovine and rat. Rat PPB was lowest as compared to that in human and bovine. Moreover, there was a decreasing trend in protein binding with an increase in

the plasma concentration for human and bovine. PPB for human and bovine was lowest at 250 µg/ml drug concentration. Paired t-test analysis of the means from the human PPB data at three different concentrations of temocillin (200, 25, and 5 µg/ml) indicated that there was a significant difference to the data for bovine (p-value=0.0005) and rat (p-value=0.0006) at all tested plasma concentrations. Similarly, PPB in bovine was found to be significantly higher as compared to rat (p-value=0.0009) at the investigated plasma concentrations (20%, 70%, and 100%).

Based on our data, we were able to identify a strong heterogeneity between species in all three antibiotics. For example, up to 3-fold difference in PPB was found for cefiderocol between humans and bovines. This difference increased up to more than 10-fold for ceftriaxone. The smallest interspecies difference was observed for temocillin at 70% and 100% human and bovine plasma concentrations, whereas this difference was found to be up to more than 10-fold when human plasma was compared to rat plasma at all tested concentrations. However, PB was always higher in humans than in the other species and consistently increased with higher plasma content.

Discussion

We examined the protein binding of three beta-lactams *i.e.* cefiderocol, ceftriaxone, and temocillin using human, bovine, and rat plasma. Our comparative protein binding across species for the beta-lactams reveals noticeable interspecies variability. This variability is critical for understanding the pharmacokinetics and optimizing dosing regimens in different clinical settings.

Our study highlights that cefiderocol's protein binding is highest in humans and varies considerably across species, with bovine showing the least protein binding and rat displaying intermediate binding. This difference becomes more pronounced at higher plasma concentrations. Our finding of low protein binding of cefiderocol increasing with plasma concentration is consistent with the research by Sato et al. (2019) where they indicated that cefiderocol exhibited a protein binding rate of approximately 58% in human plasma, which increased with higher concentrations of plasma proteins [17]. Cefiderocol PPB in rat is similar to human PPB at low drug concentrations (5 µg/ml) but decreases at higher concentrations (25 and 200 µg/ml). This suggests potential saturation

effects or differences in plasma protein affinity at varying drug levels, which could influence dosing strategies in preclinical models. The higher protein binding in humans compared to bovine and rat models also supports findings that the pharmacokinetics of cefiderocol can vary significantly across species due to differences in plasma protein composition and affinity.

Ceftriaxone is a third-generation cephalosporin known for its high protein binding, typically around 85-95% in human plasma [18]. The protein binding of ceftriaxone in our studies reveals a specific gradation of PPB among species, with humans having the highest PPB, followed by rats, and bovines having the lowest. The lower protein binding in bovine plasma compared to human and rat plasma is consistent with studies indicating species-specific differences in binding proteins such as albumin and alpha-1 acid glycoprotein (AAG) [19]. Albumin is a complex molecule with different binding sites and pockets. Our analysis indicates that cefiderocol and ceftriaxone may share similar binding sites at molecular albumin level whereas temocillin might bind to different sides in addition or instead.

The noticeable aspect of temocillin's PPB in this study is the observed decreasing trend in protein binding with increasing drug concentration for humans and bovines. Since the binding capacity of temocillin in rats is significantly diminished compared to humans this might have impact on relevance of preclinical rat models, which could be due to species-specific differences in the structure or expression of the relevant binding sites.

While our work provides valuable insights into species-specific PB profiles of beta-lactam antibiotics, there are knowledge gaps that could be addressed in future studies. In our study, we only diluted plasma, but did not account for changes in the composition during infection. AAG is a key acute-phase protein whose levels increase significantly during infection and inflammation [20]. During infection, AAG levels can rise several-fold, often exceeding 3 g/L. This increase has important implications for drug PK/PD, particularly for compounds with high affinity for AAG. Cefiderocol is predominantly bound to albumin, with a lower affinity for AAG, while ceftriaxone shows high albumin binding and moderate AAG interaction [17]. Temocillin, on the other hand,

binds to both albumin and AAG, making it more sensitive to fluctuations in these plasma proteins. The elevated AAG levels during infection could therefore reduce the free drug fraction, altering pharmacological activity. While our approach allows for a systematic investigation of protein binding, it does not replicate the dynamic protein composition observed during infection, where AAG and other acute-phase proteins are upregulated. To address these limitations, future studies could incorporate plasma from infected individuals to better mimic the conditions of infection. Moreover, future research could expand to include additional animal models to further refine our understanding of pharmacokinetic variability. Moreover, the study assumes that plasma proteins are the only binding sites for beta-lactam antibiotics. However, other tissues or compartments in the body could also contribute to drug binding, potentially influencing overall pharmacokinetic behaviour. Another facet that could provide further insights is the inclusion of pharmacodynamic experiments. While our research elucidates the extent of PB of these drugs, it does not explore how this binding translates into pharmacological effects in different plasmas.

Conclusion

By comparing cefiderocol, ceftriaxone, and temocillin, the study provides a comprehensive view of plasma protein binding across species. Since most PK/PD analyses in translational clinical studies rely on total drug concentrations and assume that the unbound fraction remains constant, this has implications for models aiming to extrapolate human efficacy from preclinical infection studies. Ultimately, understanding these PPB differences will benefit clinical trial design and clinical dosing strategies.

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Figure legends

Figure 1: Plot (mean ± SD) of PPB percentage of cefiderocol (A), ceftriaxone (B) and temocillin (C) of human (gray), bovine (purple) and rat (green) plasmas at different concentrations using ultrafiltration. The experiments were performed in duplicates. The statistical significance was determined using a sample t-test, and $p < 0.05$ was considered as significant.

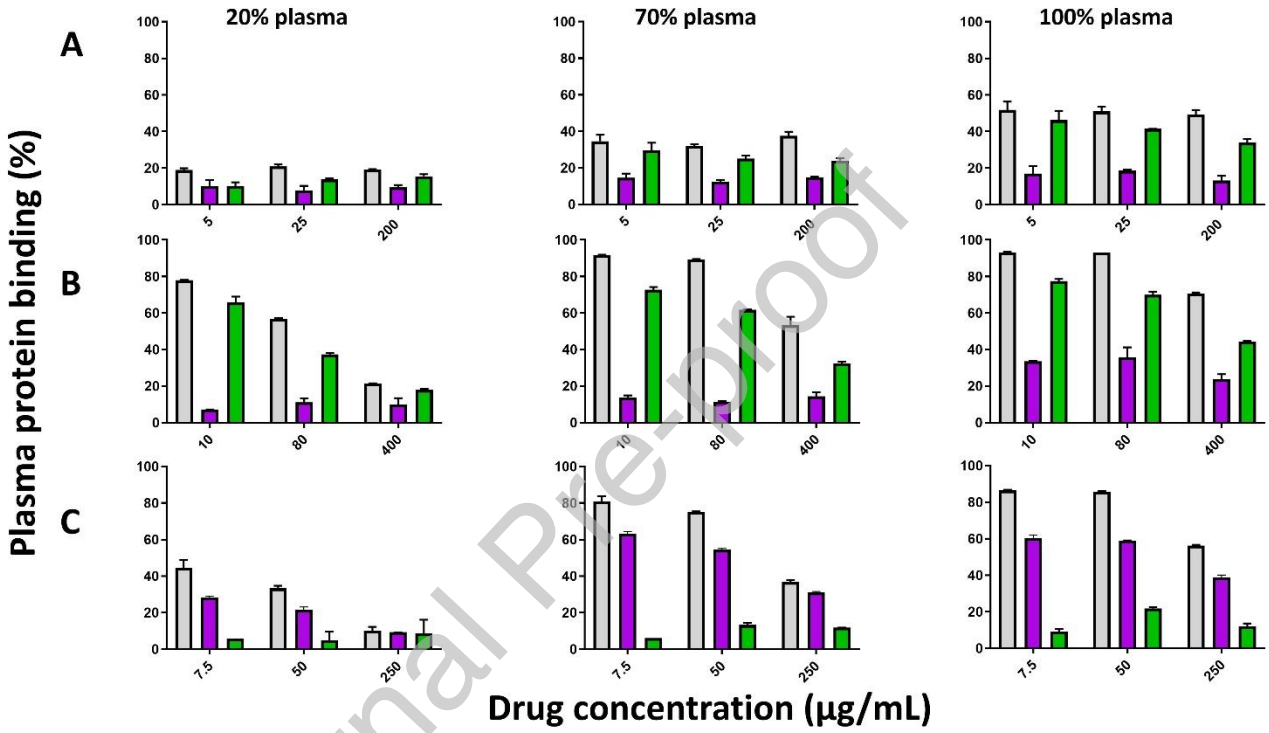

Table 1: PPB percentage of cefiderocol, ceftriaxone, and temocillin of human, bovine and rat plasmas at different concentrations using ultrafiltration. The experiments were performed in duplicate.

Drug concentration (µg/mL)	**Cefiderocol**								
	Human Plasma (%)			**Bovine Plasma (%)**			**Rat Plasma (%)**		
	20%	**70%**	**100%**	**20%**	**70%**	**100%**	**20%**	**70%**	**100%**
5	18.2 19.5	37.1 32.2	48.8 55.0	12.5 7.5	16.2 12.9	14.0 19.9	8.9 11.6	26.5 32.5	49.8 42.6
25	20.1 21.6	32.7 31.4	49.1 52.9	9.6 6.2	11.5 12.9	18.9 18.1	13.3 14.2	26.2 23.8	41.4 41.4
200	19.2 19.3	39.1 35.8	51.0 47.8	8.6 10.3	15.0 14.5	14.9 11.4	14.8 16.3	24.9 23.1	35.3 32.2
Ceftriaxone									
	Human Plasma (%)			**Bovine Plasma (%)**			**Rat Plasma (%)**		
	20%	**70%**	**100%**	**20%**	**70%**	**100%**	**20%**	**70%**	**100%**
10	78.1 78.0	91.8 91.7	93.1 93.4	7.2 7.1	12.9 14.5	33.4 33.7	68.0 63.4	73.8 71.6	78.3 76.6
80	56.5 57.2	89.1 89.4	93.2 93.2	9.6 12.7	11.5 11.6	39.6 32.0	37.8 36.7	61.3 62.0	71.2 68.8
400	21.0 21.5	49.8 56.5	70.3 71.1	12.3 7.4	16.0 12.9	22.1 25.8	18.2 17.6	31.7 33.1	44.5 43.8
Temocillin									
	Human Plasma (%)			**Bovine Plasma (%)**			**Rat Plasma (%)**		
	20%	**70%**	**100%**	**20%**	**70%**	**100%**	**20%**	**70%**	**100%**
7.5	47.5 41.4	82.8 78.8	86.2 86.7	27.8 28.8	64.0 62.0	59.1 61.5	5.7 6.1	6.0 5.7	10.1 7.9
50	32.4 34.3	75.2 75.4	85.2 86.0	20.3 22.6	53.8 54.9	59.2 58.7	1.6 8.3	12.4 14.1	22.3 21.4
250	11.6 8.4	36.1 37.6	56.0 56.5	9.4 9.2	30.6 31.4	39.7 37.9	3.3 13.9	11.8 11.7	11.1 13.2