Plasma protein binding of fluoroquinolones affects antimicrobial activity

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Received 12 October 2007; returned 3 December 2007; revised 7 December 2007; accepted 10 December 2007

Objectives: In contrast to most antimicrobial classes, there is a doubt about the impact of protein binding (PB) on the antimicrobial activity of fluoroquinolones. We set out to evaluate the suitability of previously used models for investigating the influence of PB on bacterial killing by fluoroquinolones.

Methods: PB of moxifloxacin and trovafloxacin was determined in Mueller–Hinton broth (MHB) containing different concentrations of human serum or albumin. Bacterial growth curves of Staphylococcus aureus and Pseudomonas aeruginosa were determined in pure serum, pure MHB and MHB containing different amounts of serum or albumin. Killing of both strains at concentrations equal to the MIC was investigated for moxifloxacin and trovafloxacin in MHB and also in medium that showed PB values identical to those of pure serum.

Results: Frequently used media for investigating the impact of PB, i.e. MHB containing 20% to 70% serum or 4% albumin, did not reach the level of PB achieved in pure serum or significantly hampered bacterial growth compared with pure MHB. PB in MHB containing 12% albumin was identical to that in pure serum but did not impair bacterial growth. Addition of 12% albumin significantly reduced bacterial killing by both fluoroquinolones compared with that found in pure MHB.

Conclusions: For fluoroquinolones, standard media might be insufficient to investigate the impact of PB on bacterial killing. MHB containing 12% albumin seems to be a promising medium in this context. For moxifloxacin and trovafloxacin, PB leads to significant reduction of antimicrobial activity.

Keywords: albumin, serum, trovafloxacin, moxifloxacin, in vitro

Introduction

The importance of protein binding (PB) in antibacterial efficacy is well documented for all antimicrobial classes, based on its effects on tissue penetration, elimination half-life and the volume of distribution.1–3 In contrast, no general consensus has been reached to date as to whether PB also impacts antimicrobial activity by reducing the available fraction of free drug.3,4 Although the inhibiting effect of PB on bacterial killing is generally accepted for β-lactams, doubts about the usefulness of extrapolation of findings from β-lactams to fluoroquinolones persist.3–10 These doubts are mainly based on the lack of reduction of antibacterial activity after addition of protein to previously protein-free growth media.11–13 Commonly, the impact of PB is investigated by addition of serum at concentrations between 20% and 70% or by addition of albumin at 4% to Mueller–Hinton broth (MHB).11–14 However, most studies neither measured PB in their specific setting nor investigated differences in bacterial growth between broth and media containing high amounts of serum. Thus, it cannot be excluded that reduced bacterial killing by fluoroquinolones is masked by impaired bacterial growth in media containing serum.

This study, therefore, was set out to investigate two possible methodological pitfalls of in vitro models used to investigate the impact of PB on antimicrobial killing of fluoroquinolones, namely: (i) differences between PB in test media and in pure serum; and (ii) the correlation of bacterial growth in test media to that seen in pure MHB. The optimal test medium would on the one hand achieve a level of PB comparable to that of pure serum, but would on the other hand not influence bacterial
growth compared with pure broth. We have investigated the currently used media with regard to both goals for fluoroquinolones and have aimed at developing an optimized medium. The novel medium was used to re-evaluate the influence of PB on the activity of fluoroquinolones.

Time–kill curves were employed in the present study, since they correlate excellently with in vivo efficacy and have been previously recommended to investigate the impact of PB on antimicrobial activity.\textsuperscript{5,16} For this purpose, we have selected \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}, two relevant representatives of Gram-positive and -negative bacteria. Moxifloxacin and trovafloxacin were used as model fluoroquinolones with moderate (~40%) and high (~75%) PB, respectively.\textsuperscript{3,4}

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\section*{Materials and methods}

\subsection*{Organisms}

\textit{S. aureus} (ATCC 29213) and \textit{P. aeruginosa} (ATCC 27853) were obtained from the American Type Culture Collection. Between experiments, strains were stored in liquid nitrogen at −196°C.

\subsection*{Antibiotics and growth media}

Moxifloxacin was obtained from Pfizer (USA) and trovafloxacin was obtained from Pfizer (USA). Both antimicrobial agents were prepared and stored throughout the investigations following the manufacturers’ recommendations. Columbia agar plates (Columbia + 5% sheep blood; Heipha, Germany) and MHB (Oxoid Ltd, UK) were used as growth media for bacteria. Human albumin was obtained from Behring (Human Albumin ‘Behring’ 20%, Germany). Pooled human serum was obtained from Sigma-Aldrich (Germany).

\subsection*{In vitro susceptibility tests}

MIC values were determined by the 2-fold serial broth microdilution method, according to the CLSI (formerly NCCLS) criteria.\textsuperscript{17} Therefore, \textit{S. aureus} and \textit{P. aeruginosa} were pre-cultured overnight on a Columbia agar plate and were then introduced at an initial inoculum of ~5 × 10\textsuperscript{8} cfu/mL into MHB. Growth media contained defined concentrations of moxifloxacin or trovafloxacin in decreasing 2-fold steps. The lowest concentration of antibiotic that inhibited visible bacterial growth after 20 h of incubation at 37°C was defined as the respective MIC.

\subsection*{Determination of PB}

PB of moxifloxacin and trovafloxacin was assessed in pure MHB, 100% serum, MHB containing 20% and 70% serum and MHB containing 4%, 8%, 12% and 16% albumin by use of the ultrafiltration method. For each medium, drug concentrations of 0.2, 1 and 5 mg/L were investigated, which are representative of the concentrations of moxifloxacin and trovafloxacin achieved in serum (maximal concentrations 4.34 and 2.09 mg/L, respectively).\textsuperscript{18} The investigated mixtures were incubated at 37°C for 30 min to allow PB to take place. Consecutively, 0.3 mL aliquots of each mixture were transferred into a centrifugal filter unit equipped with a low-binding regenerated cellulose membrane (Ultrafree-MC\textsuperscript{TM}, nominal molecular weight limit 5000; Millipore Corp., USA) and ultrafiltered by centrifugation at 1400 g for 30 min at room temperature. Non-specific binding of trovafloxacin and moxifloxacin to the filter membrane during ultrafiltration was assessed in Ringer’s solution (Mayerhofer Pharmazeutika, Austria) at each investigated concentration. Drug concentrations in ultrafiltrates (\(C_{\text{UF}}\)) were corrected for the mean non-specific binding.

PB was calculated using the formula:

\[
\text{PB} \ (% ) = 100 - 100 \frac{C_{\text{UF corr}}}{C_{\text{pre-UF}}}.
\]

where \(C_{\text{UF corr}}\) is \(C_{\text{UF}}\) corrected for non-specific binding and \(C_{\text{pre-UF}}\) is the concentration before ultrafiltration. Each ultrafiltration experiment was performed in duplicate.

\subsection*{Chemical analysis}

Samples were analysed in duplicate using a validated high-performance liquid chromatography method with modifications.\textsuperscript{19} Pure ciprofloxacin (internal standard) was obtained from Bayer; all other reagents were purchased from Sigma-Aldrich. Calibration standards were prepared daily by spiking each matrix with trovafloxacin and moxifloxacin, respectively, to concentrations ranging from 0.04 to 8 mg/L. Protein-containing samples were de-proteinized by methanol precipitation. Ultrafiltrates were analysed without further preparation. The injection volume was 15 μL. Isocratic separation was performed at 45°C on a Hypersil BDS C18 column (150 × 2.1 mm, particle size 5 μm; Hypersil-Keystone, UK). The mobile phase consisted of 16 mM ortho-phosphoric acid in water adjusted to pH 3 with tetrabutyl ammonium hydroxide and acetonic acid (volume ratio 94:6). The flow rate was 0.4 mL/min. The spectrophotometer was set at 272/450 nm (excitation/emission wavelength) for trovafloxacin and at 293/500 for moxifloxacin. Coefficients of accuracy and precision were <7%.

Medium pH was measured by use of a microprocessor pH-meter (WTW, Germany) at 37°C. Electrolyte concentrations (sodium, potassium, chloride, calcium and magnesium) in test media were determined at the routine laboratory of the General Hospital of Vienna. Likewise, total protein and albumin in purchased serum were determined at the routine laboratory.

\subsection*{Bacterial growth curves}

\textit{S. aureus} and \textit{P. aeruginosa} were pre-cultured overnight on a Columbia agar plate and were afterwards introduced at an initial inoculum of ~5 × 10\textsuperscript{8} cfu/mL into MHB, pure serum and MHB containing 20% serum, 70% serum or 12% albumin. Culture tubes containing 3 mL aliquots were kept in a water bath at 37°C. Bacteria were counted at 0, 4 and 8 h of incubation. After vortexing the culture tubes, two 50 μL samples were removed and serially diluted with 0.9% sodium chloride. After each dilution step, 20 μL was plated onto Columbia agar plates, which were incubated for 24 h at 37°C. Afterwards, the colonies were counted and back extrapolated to the original volume to determine cfu/mL. Each experiment was performed five times.

To assess whether albumin was consumed by \textit{S. aureus} or \textit{P. aeruginosa} during the 24 h observation time, albumin concentrations in MHB containing 12% albumin were determined at baseline and after 24 h of incubation with bacteria. Measurement of albumin was performed at the routine laboratory of the General Hospital of Vienna.
Time–kill curves

Bacterial killing curves were determined by inoculating *S. aureus* and *P. aeruginosa* with antibiotic concentrations equal to the MIC in MHB and MHB containing 12% albumin. In addition, killing curves were performed in pure MHB containing only the calculated free concentration of the antibiotic in serum (MHB<sub>calculated free</sub>). The free fraction of the antibiotic in serum was calculated as follows:

\[
\text{concentration}_\text{calculated free} = \frac{\text{concentration}_\text{total}}{(100 - \text{PB}_{\text{serum}} \text{ in } \%)/100}
\]

In brief, antibiotic concentrations in the flask were adjusted in MHB or MHB containing albumin according to the desired concentration. Culture tubes containing 3 mL aliquots were kept in a water bath at 37°C to allow PB to take place. After 30 min, tubes were inoculated with *S. aureus* or *P. aeruginosa* at an approximate inoculum of \(5 \times 10^5\) cfu/mL. Samples were drawn and bacteria were counted at 0, 4 and 8 h of incubation at 37°C as described above.

Each simulation was performed five times. For each test, medium growth controls were performed without addition of antibiotic. Bacterial killing was expressed as log<sub>10</sub> differences in bacterial cfu/mL between the initial inoculum and 8 h after exposure.

Statistical calculations

For statistical analysis, Mann–Whitney U-tests were performed by using a commercially available computer program (Statistica<sup>®</sup>, StatSoft, Inc., Tulsa, OK, USA). A two-sided *P* value <0.05 was considered as the level of significance. In all figures, data are depicted as means ± SD.

Results

In vitro susceptibility tests

MIC values for *S. aureus* were 0.06 and 0.03 mg/L and those for *P. aeruginosa* were 2 and 0.5 mg/L for moxifloxacin and trovafloxacin, respectively. All values were within CLSI ranges.<sup>17</sup>

Chemical analysis of media

Chemical analysis of pure serum used in the present study revealed a typical pattern of protein composition (70.8 g/L total protein and 42.2 g/L albumin). The pH in MHB containing 4% albumin was lower than the pH in serum (6.8 and 8.0, respectively). Concentrations of sodium, chloride and magnesium did not differ between these media. However, the concentrations for potassium (3.4 versus 1.9 mM) and calcium (2.3 versus 1.1 mM) were 2-fold higher in pure serum compared with MHB containing 4% albumin.

PB of moxifloxacin and trovafloxacin in different media

In agreement with previously published data, PB in pure serum was 38 ± 4.2% and 77.9 ± 2.7% for moxifloxacin and trovafloxacin, respectively.<sup>3,4</sup> Non-specific binding to the cellulose membrane of the centrifugal filter was low and not concentration-dependent for moxifloxacin and trovafloxacin with values of 6.5 ± 2.1% and 5.0 ± 3.6%, respectively. Likewise, PB was not concentration-dependent for any test medium within the investigated concentration range of 0.2–5 mg/L for both antibiotics. Thus, for each investigated medium, PB at 0.2, 1 and 5 mg/L (each investigated in duplicate) is shown as mean ± SD (n = 6) in one bar in Figure 1.

For moxifloxacin, PB in pure MHB, MHB containing 4% or 8% albumin and MHB containing 20% or 70% serum was significantly lower (*P < 0.05*) compared with pure serum. On the other hand, PB in MHB containing 16% albumin was significantly higher compared with 100% serum (*P < 0.05*). Only for MHB containing 12% albumin was no relevant difference compared with pure serum observed (Figure 1a).

Similarly, for trovafloxacin all media except MHB containing 12% and 16% albumin demonstrated significantly lower PB than 100% serum (Figure 1b). Hence, with regard to PB for both investigated fluoroquinolones, MHB containing 12% albumin was the only medium which did not show a significant difference compared with pure serum.

![Figure 1](https://academic.oup.com/jac/article-abstract/61/3/561/726499/1a.png)
Bacterial growth in different media

Mean (n = 5) bacterial growth profiles of *S. aureus* and *P. aeruginosa* in MHB, pure serum and MHB containing 20% serum, 70% serum or 12% albumin are depicted in Figure 2. Data were consistent for both bacterial strains. MHB containing 12% albumin was the only tested medium that did not result in a significant difference with regard to bacterial counts compared with pure MHB at any time point.

Measurements of albumin concentrations at baseline and at the end of the growth experiments did not show evidence of consumption of albumin by *S. aureus* or *P. aeruginosa* within 24 h.

Time–kill curves

Figure 3 presents mean (n = 5) bacterial time–kill profiles of *S. aureus* and *P. aeruginosa* after exposure to moxifloxacin or trovafloxacin at concentrations equal to the MIC. Data are shown for killing curves in MHB, MHB containing 12% albumin and MHB<sub>calculated</sub> free. Mean differences in bacterial log<sub>10</sub> cfu/mL between the initial inoculum and 8 h after exposure are listed in Table 1.

Bacterial killing by moxifloxacin in MHB containing 12% albumin was slightly reduced by 0.75 log<sub>10</sub> cfu/mL for *P. aeruginosa* but markedly reduced by 2.11 log<sub>10</sub> cfu/mL for *S. aureus* compared with that in pure MHB. For trovafloxacin, addition of 12% albumin to MHB led to even higher impairment of bacterial killing, resulting in a reduction of bacterial killing by 3.48 and 2.06 log<sub>10</sub> cfu/mL for *S. aureus* and *P. aeruginosa*, respectively.

Antimicrobial activity of MHB containing 12% albumin was in good agreement with activity for MHB<sub>calculated</sub> free as indicated by killing curves that closely reflect each other (Figure 3).

Discussion

For fluoroquinolones, doubts on the effect of PB on antimicrobial activity are mainly based on the lack of change of MIC values after addition of 4% albumin or different amounts of serum to growth media. However, the present study revealed that these media have substantial limitations for investigating the influence of PB on bacterial killing by fluoroquinolones. For moxifloxacin and trovafloxacin, neither addition of 20% serum nor 4% albumin achieved an extent of PB comparable to that of serum (Figure 1). Adding 70% serum achieved PB comparable to pure serum for trovafloxacin, only. However, both pure serum and MHB containing 70% serum considerably impaired bacterial growth (Figure 2). Therefore, MHB containing any amount of serum was not considered an appropriate medium for investigating the impact of PB.

In order to develop a more appropriate test medium, albumin concentrations were up-titrated until a satisfactory level of PB was achieved. MHB containing 12% albumin (corresponding to 114 g/L albumin) showed PB congruent with pure serum but did not impair bacterial growth of *S. aureus* and *P. aeruginosa* compared with pure MHB over 24 h. MHB containing 12% albumin was, therefore, employed in consecutive time–killing curves.

By addition of 12% albumin to MHB, killing of both *S. aureus* and *P. aeruginosa* was significantly reduced by 1–3 log<sub>10</sub> cfu/mL after 8 h of exposure to fluoroquinolones at concentrations equal to the MICs (Figure 3 and Table 1). Addition of albumin had higher impact on antimicrobial activity of trovafloxacin than of moxifloxacin. This finding may reflect the higher PB of trovafloxacin of 78% compared with that of moxifloxacin of 38%, which is in close agreement with previous findings observed for β-lactam antibiotics.

Only small differences were observed between the time–killing curves in MHB containing the calculated free fraction of antibiotic in serum and those in MHB containing 12% albumin. This observation underlines the concept that impairment of antimicrobial activity by PB mainly relies on reduction of the available free fraction of antibiotic. Interestingly, impairment of antimicrobial killing by addition of albumin was more pronounced for *S. aureus* than for *P. aeruginosa*. Although differences in killing kinetics between Gram-positive and -negative strains have been described for various conditions, variation in
Modified method to assess impact of protein binding

Figure 3. Bacterial time–kill profiles of moxifloxacin (a and b) and trovafloxacin (c and d) in media containing or lacking albumin. *S. aureus* (a and c) and *P. aeruginosa* (b and d) were exposed to concentrations equal to the MIC in MHB or MHB containing 12% albumin. MIC values for *S. aureus* were 0.06 and 0.03 mg/L and those for *P. aeruginosa* were 2 and 0.5 mg/L for moxifloxacin and trovafloxacin, respectively. In addition, bacteria were grown in MHB containing the calculated free fraction of antibiotic in serum. Growth controls without addition of antibiotic are presented for MHB and MHB containing 12% albumin. Data are shown as means ± SD (*n* = 5).

Table 1. Differences in bacterial counts (log_{10} cfu/mL) for *S. aureus* and *P. aeruginosa* between the initial inoculum and 8 h after exposure to moxifloxacin or trovafloxacin

<table>
<thead>
<tr>
<th></th>
<th>MHB</th>
<th>MHB + 12% albumin</th>
<th>MHBcalculated free</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moxifloxacin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>−2.29 ± 0.22</td>
<td>−0.19 ± 0.12</td>
<td>0.59 ± 0.18</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.03 ± 0.12</td>
<td>0.78 ± 0.22</td>
<td>0.34 ± 0.15</td>
</tr>
<tr>
<td><strong>Trovafloxacin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>−1.61 ± 0.19</td>
<td>1.87 ± 0.09</td>
<td>2.35 ± 0.11</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>−0.39 ± 0.16</td>
<td>1.67 ± 0.18</td>
<td>1.70 ± 0.16</td>
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</tbody>
</table>

Data are presented as means ± SD for MHB and MHB containing 12% albumin and for MHB containing the calculated free fraction of antibiotic in serum.

The impact of PB for individual strains demands further investigation.20,21

Analysis of the media used in the present study showed that pure serum and MHB containing 4% albumin contain identical amounts of albumin. Yet, PB in 4% albumin did not achieve the level of binding of fluoroquinolones observed in pure serum. This finding might be explained either by differences in the binding potency of albumin within these environments or by binding of fluoroquinolones to serum constituents beside albumin. Binding of a drug to albumin is dependent on the drug concentration, the concentration of albumin, the number of binding sites and the association constant.22 Neither the concentrations of drugs nor of albumin differed between serum and MHB containing 4% albumin and differences in binding sites can be largely excluded. Thus, only a difference in the association constant, i.e. the factor that describes the affinity of a drug to a binding site, could explain the variation in the binding of fluoroquinolones to albumin contained in serum or MHB.
Human albumin consists of three homologous domains that undergo several well-described proton-induced conformational changes. One of these pH-dependent transformations occurs at the pH range of 6–9 and is referred to as the neutral-to-base (N–B) transition. It has been shown that calcium ions at physiological concentrations induce N–B transition at lower pH values. While many substances have stronger affinity to the N-isofrom some show higher affinity to the B-isofrom of albumin. In the present study, both pH (8 versus 6.8) and concentration of calcium ions (2.3 versus 1.1 mM) were higher in serum than in MHB containing albumin, possibly resulting in a higher prevalence of the B-isofrom in serum. Although the impact of the N–B transition on the binding of fluoroquinolones is unexplored, this might deliver an explanation for the varying binding properties of serum- and albumin-containing media observed in this study.

In addition, although plasma PB of fluoroquinolones is usually exclusively ascribed to albumin, there is evidence that some fluoroquinolones may bind to a range of other proteins including transferrin, lactoferrin and α-1-acid glycoprotein. Thus, binding to serum proteins except albumin may deliver another explanation for the discrepancy in fluoroquinolone binding between serum and MHB containing albumin at physiological concentrations.

The fact that only two representatives of the class of fluoroquinolones were investigated with regard to PB and its impact on bacterial killing is a clear limitation of the present study. Further studies seem necessary to explore whether a strict correlation between percentage of PB and reduction of antimicrobial activity, as previously established for β-lactams, also exists for fluoroquinolones.

In conclusion, a significant impact of PB on antimicrobial activity of moxifloxacin and trovafloxacin was observed in the present study. For fluoroquinolones, previously employed media for investigation of this question might be insufficient, since these media impair bacterial growth compared with MHB and/or do not achieve the level of PB observed in pure serum. In this context, MHB containing 12% albumin is a promising medium as it did not display either of these limitations in the present study.

Acknowledgements

We would like to thank Christian Joukhadar, MD, for discussion during the preparation of the manuscript and Friederike Traumnüller, MD, for performing the chemical analyses.

Funding

No specific funding was received for this study.

Transparency declarations

None to declare.

References


Modified method to assess impact of protein binding


