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Binding of temocillin to plasma proteins *in vitro* and *in vivo*: the importance of plasma protein levels in different populations and of co-medications

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Background: Temocillin plasma protein binding (PPB) in healthy individuals is reported to be ~85% but had not been studied in patients.

Objectives: To obtain normative data on temocillin PPB in patients in relation to infection and impact of co-medications widely used in ICU.

Methods: Plasma was obtained from healthy individuals (Group #1), non-ICU patients with UTI (Group #2), ICU patients with suspected/confirmed ventriculitis (Group #3) or with sepsis/septic shock (Group #4). Total and unbound temocillin concentrations were measured in spiked samples from temocillin-naive donors (*in vitro*) or in plasma from temocillin-treated subjects (*in vivo*). The impact of diluting plasma, using pharmaceutical albumin, or adding drugs potentially competing for PPB was tested in spiked samples. Data were analysed using a modified Hill–Langmuir equation taking ligand depletion into account.

Results: Temocillin PPB was saturable in all groups, both *in vitro* and *in vivo*. Maximal binding capacity (B_{max}) was 1.2–2-fold lower in patients. At 20 and 200 mg/L (total concentrations), the unbound fraction reached 12%–29%, 23%–42% and 32%–52% in Groups #2, #3, #4. The unbound fraction was inversely correlated with albumin and C-reactive protein concentrations. Binding to albumin was 2–3-fold lower than in plasma and non-saturable. Drugs with high PPB but active at lower molar concentrations than temocillin caused minimal displacement, while fluconazole (low PPB but similar plasma concentrations to temocillin) increased up to 2-fold its unbound fraction.

Conclusions: Temocillin PPB is saturable, 2–4-fold lowered in infected patients in relation to disease severity (ICU admission, hypoalbuminaemia, inflammation) and only partially reproducible with albumin. Competition with other drugs must be considered for therapeutic concentrations to be meaningful.

Introduction

Temocillin, a β-lactam originally described as showing stability to many β-lactamases, is a useful carbapenem-sparing drug in the context of widening ESBL-mediated resistance in Enterobacterales.[1] It shows high plasma protein binding (PPB; 80%–85%), a prolonged half-life (~4 h), adequate penetration and activity in extravascular spaces.[2–4] Its high and self-saturable PPB at clinically achievable concentrations,[5,6] however, complicates dose optimization based on pharmacokinetic/pharmacodynamic (PK/PD) concepts, as only the unbound forms of β-lactams are generally considered active.[7] The high PPB reported for temocillin, however, came from a single study with healthy volunteers,[2] while clinical studies with ICU patients documented much lower mean values (approximately 45%) but high interpatient variability.[8,9] This situation is shared by flucloxacillin, another β-lactam with high PPB.[10]

Our aim was therefore to characterize temocillin PPB in detail and to contribute to establishing the reasons for interpatient variability. To reach this goal, we analysed samples from healthy subjects and patients with infections of increasing severity. We developed an equation for estimating drug-binding parameters that takes ligand depletion into account.[11–14] We then used it to describe temocillin PPB in our samples, in relation to the degree of infection severity and inflammation of the subjects. We also examined the potential impact on temocillin-binding parameters of other drugs frequently administered to ICU patients and with variable degrees of PPB. In a nutshell, we found that (i) the binding capacity of temocillin is markedly decreased in infected patients, probably due to inflammation and ensuing hypoalbuminaemia; (ii) pharmaceutical albumin does not allow reproduction of the saturable character of temocillin binding in plasma; and (iii) drugs with high PPB cause only minimal displacement when tested at their clinically relevant concentrations if these are lower than those of temocillin.

Materials and methods

Drugs and reagents

Temocillin was provided by EUMEDICA S.A. (Manage, Belgium) as Negaban®.[15] All other drugs or reagents were of suitable quality and obtained from traceable origins (see details in the Supplementary Materials and methods, available as Supplementary data at *JAC* Online).

Collection of samples

Samples were collected from healthy individuals (Group #1), patients with urinary tract infection (UTI) hospitalized in common wards [Group #2; Algemeen Ziekenhuis Delta (AZ-Delta), Roeselare, Belgium] and ICU patients (Cliniques universitaires St-Luc, Brussels, Belgium) with documented/suspected brain ventriculitis (Group #3) or with sepsis/septic shock (SOFA score >6;[16] Group #4). Biological parameters are shown in Table 1 and Table S1. Half of the samples were obtained from temocillin-naive donors [used for spiking studies (*in vitro*)], and the other half from 14 volunteers (8 participating in a previous temocillin PK study;[6] 6 recruited in Austria with authorization from the ethical committee) or from temocillin-treated patients (see Figure S1 for doses and schedules).

Preparation and handling of samples; biochemical assays

Plasma was collected after blood centrifugation and frozen at −80°C. After thawing, its pH was at or very close to 7.2. For *in vitro* studies, samples from donors were mixed with small volumes (≤3% of total) of concentrated temocillin solutions (plus a competing drug in dedicated studies) to reach the desired final concentrations and incubated at 37°C for 30 min before assay. Sample preparation for experiments looking at matrix dilution effects is explained in the Supplementary Materials and methods. Validated automated methods (see details in Supplementary Materials and methods) were used for the assay of total proteins, albumin or C-reactive protein (CRP).

Determination of total, unbound and bound temocillin concentrations

Total and unbound temocillin concentrations were measured after protein precipitation with methanol and on centrifuge-generated ultrafiltrates (exclusion: 30 kDa), respectively, using a previously validated HPLC-MS/MS method for analysis in serum[18] or plasma[6] samples. All temocillin-spiked samples were incubated at 37°C for 30 min (a time sufficient to reach equilibrium of drug PPB[11] and used in most studies).[19] Lack of binding of temocillin to surfaces of the ultrafiltration devices and negligible influence of temperature during the centrifugation step [at 25°C (as in all published studies with temocillin PPB) versus 37°C (often selected in PPB studies for other drugs)] were demonstrated using matrix analysis approaches (see Supplementary Materials and methods, Table S2 and Figure S2A–C). Bound concentrations were calculated as the difference between total and unbound concentrations, and the unbound fraction as the ratio between the unbound and the total concentrations.[5,13]

Impact of drugs with high PPB, and of tacrolimus and fluconazole, on temocillin PPB

Diazepam, midazolam, propofol and pantoprazole, all reported as highly protein bound [>95%; low dissociation constant ($<10^{-4}$ M); see Table S3], were selected based on their frequent use in patients hospitalized in ICU (based on local hospital pharmacy data). Tacrolimus was added because of reported interferences with anti-infective therapy, and fluconazole as a drug with lower PPB (see Table S3 for additional details). To ensure clinical significance, drugs were added at their clinically pertinent concentration[20] to plasma from Group #1 donors together with temocillin (see Supplementary Materials and methods for more details).

Model for the study of temocillin binding

The Hill–Langmuir equation (H–Le), used in most ligand–receptor studies undertaken in a molecular pharmacology context, plots the bound and unbound ligand concentrations as dependent and independent variables. It assumes that binding is minimal and that the free concentration is almost equal to the total concentrations.[21] None of these conditions are met in temocillin PPB studies. First, the H–Le is invalid in situations of ligand depletion, which takes place when binding is not minimal and when the unbound concentration becomes much lower than the total concentration, a situation known as ligand depletion. Second, the bound concentrations of drugs are never measured in drug PPB studies and the difference between the total and unbound concentrations is used as a surrogate. Inspired by previous pharmacological studies,[22] we developed an explicit form of the H–Le in which the temocillin bound concentration remains the dependent variable but is expressed as a function of its total concentration (Equation 1):

Table 1. Plasma total protein, plasma albumin and CRP concentrations in study populations

	Study populations			
	Healthy individuals	General ward patients[a]	ICU patients[a]	
Parameter	Group #1	Group #2	Group #3	Group #4
Proteins				
Donors (unexposed to temocillin; samples used for spiking experiments)				
Number	14	7	9	7
Plasma total protein (g/L)[b]	77.1 ± 4.58 (A;a)	63.3 ± .28 (B;a)	63.8 ± 4.65 (B;a)	37.7 ± 5.10 (C;a)
Plasma albumin (g/L)[c]	47.42 ± 3.547 (A;a)	33.7 ± 3.49 (B;a)	34.8 ± 2.89 (B;a)	17.9 ± 1.90 (C;a)
Subjects [having received temocillin (healthy volunteers) or treated with temocillin (patients)]				
Number	14[d]	12	7	14
Plasma total protein (g/L)[b]	70.9 ± 3.04 (A;b)	64.7 ± 6.59 (B;a)	64.5 ± 4.35 (B;a)	48.9 ± 8.90 (C;b)
Plasma albumin (g/L)[c]	43.9 ± 2.51 (A;b)	31.1 ± 4.26 (B;a)	32.6 ± 4.52 (B;a)	19.1 ± 3.98 (C;a)
CRP				
Number	[e]	11	7	14
Median (min–max)		82.4 (13.5–328)	74.0 (14.7–131)	179 (50.9–550)
$\log_{10}$ mean ± SD (mg/L)[f]		1.86 ± 0.358 (B)	1.73 ± 0.31 (B)	2.23 ± 0.339 (C)

Statistical analysis by row: comparing all values, unpaired ordinary analysis of variance [ANOVA (with *P* value at the end of each row)] followed by *post hoc* Tukey's multiple comparison test [values with different upper-case letters (A, B, C) are significantly different from each other ($P < 0.001$)]; if with the same letter, the difference is not significant ($P > 0.05$). In Table 1, A is always given to the data in the left column. Statistical analysis by column: unpaired *t*-test comparing *in vitro* and *in vivo* data. Values with different lower-case letters (a, b) are significantly different from each other (highest *P* value observed: <0.02); if with the same letter, the difference is not significant ($P > 0.05$). In Table 1, a is always given to the data closest to the top of the table.

[a]Patients with documented UTI hospitalized in the Department of Urology of the AZ Delta Ziekenhuis (Group #2); patients hospitalized in ICU at Cliniques universitaires St-Luc with suspected/documented ventricular infection (Group #3); or sepsis/septic shock (Group #4) (see Table S1).

[b]Local normal values: 64–83 g/L (Cliniques universitaires St-Luc).

[c]Local normal values: 35–52 g/L (Cliniques universitaires St-Luc).

[d]This includes the eight volunteers enrolled in a pharmacokinetic study[6] plus six volunteers recruited in Austria (with suitable modification of the protocol accepted by the Ethical Committee).

[e]No assay was made for healthy individuals but local normal and published values[17] are <5 mg/L.

[f]Untransformed values showed inconsistent results in test for normality of distribution. Log-transformed data showed normality and were therefore used for further statistical analysis.

$$y = \frac{\left(-(K_d + x + B_{max}) + \sqrt{((K_d + x + B_{max})^2 - (4 \times x \times B_{max}))}\right)}{-2} \quad (1)$$

where y is the concentration of bound temocillin, x is the measured total temocillin concentration of each sample, and K_d and B_{max} are the binding parameters. This equation will be referred to as the 'saturation binding curve accounting for ligand depletion' (SBCALD); its slope factor (Hill coefficient) was set to 1, as in the starting H–Le (see Appendix in the Supplementary data for details of its construction and performance).

Data and statistical analysis

All datasets (and their transformants, if suitable) were checked for distribution normality before performing parametric statistics or regressions and curve-fitting analyses (details in Supplementary Materials and methods). Non-parametric statistics were used for analysis of non-normal distributions or in the case of uncertainties. Differences were considered as significant for *P* values of <0.05. Prism version 9.2/9.4 (GraphPad Software, San Diego, CA, USA) was used throughout.

Ethical approval and study registration

The *ad hoc* authority of each clinical institution approved and registered the study protocol [AZ Delta: Commissie medische ethiek (no. B403201938914); CU St-Luc: Comité d'Ethique hospitalo-facultaire (no. 1737/2015); Medical University of Vienna: Ethic Committee (no. 1737/2015)], which was then globally approved and given a unique Belgian registration no. (B403201629439) by the Comité d'Ethique hospitalo-facultaire of the Health Sciences Sector of the Université catholique de Louvain. The study protocols were registered at ClinicalTrials.gov (NCT03440216 and NCT03557840) and EudraCT (number 2015-003457-18).

Results

Unbound temocillin in plasma: in vitro and in vivo studies

We first measured the concentration of unbound temocillin as a function of its total concentration in plasma samples obtained from (i) donors and spiked with temocillin (*in vitro* studies); and (ii) subjects having received temocillin prior to collection (*in vivo* studies). All samples were stratified into four groups, based on their health status (from healthy to critically ill patients; see

Table 1 and Table S1 for details). Results are shown graphically in Figure 1 (numerical data and analyses in Table 2 and Table S4.1).

In vitro [Figure 1(a–c)], unbound temocillin concentration could be related to its total concentration using a quadratic polynomial function, demonstrating a saturable character of temocillin PPB in spiked samples from all four groups (Figure 1a). Unbound temocillin concentration increased stepwise from Groups #1 to #4, with statistically significant and consistent increases of the regression parameters of the fitted quadratic functions (Table S4.1.A.1). The temocillin unbound fraction increased linearly as a function of its total concentration (Figure 1b). Slopes (B1 parameter) were close to each other, but offset values (B0 parameter; starting at a total concentration of 20 mg/L) increased in a statistically significant, stepwise and consistent fashion from Group #1 to #4 (Table S4.1.B.1), with unbound fractions reaching, respectively, 5%–18%, 18%–35%, 25%–45% and 35%–58% for Groups #1, #2, #3 and #4, for 20–200 mg/L total concentrations. Figure 1c shows the fitting of the proposed SBCALD function to the data from each group (Table 2.a1 for calculated binding parameters). K_d was increased 1.4–2.2-fold in patients compared with healthy individuals but with no relation to the type of infection. Conversely, B_{max} consistently decreased from 294 mg/L in Group #1 to 240, 179 and 108 mg/L in Groups #2, #3 and #4, with statistically significant differences between each group.

In vivo [Figure 1(d–f)], similar observations were made, namely (i) saturation of temocillin PPB in samples from all four groups; (ii) stepwise and consistent increases of the unbound temocillin

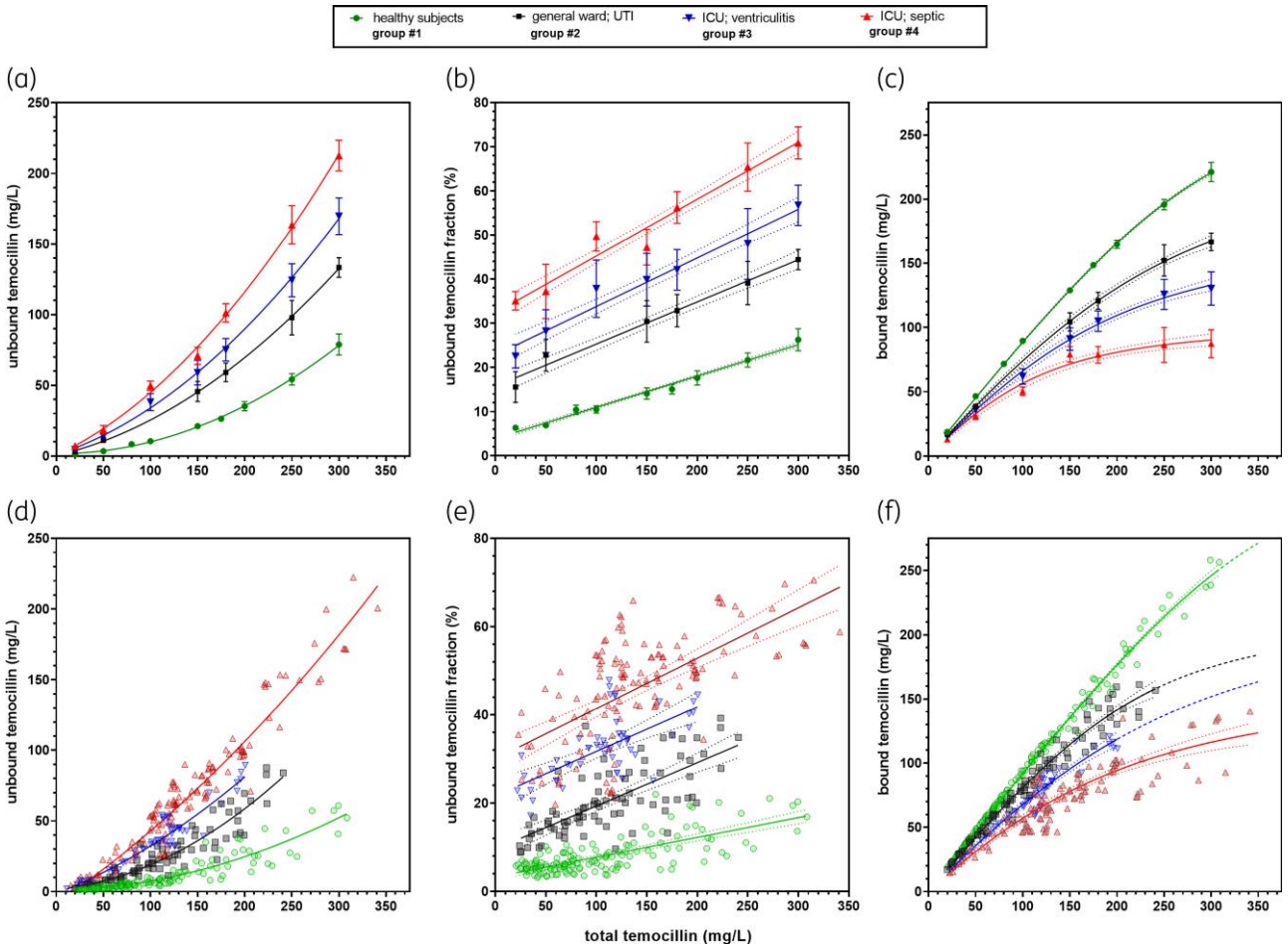

Figure 1. Binding of temocillin in plasma samples. (a–c) *In vitro* studies (spiked samples); data are shown as mean ± SD (number of individuals = 14, 7, 9 and 7). (d–f) *In vivo* studies (samples from subjects or patients having received temocillin). Each data point corresponds to a unique sample, but samples obtained from individuals in each of the four groups (n = 14, 12, 7 and 14) share the same symbol and colour. Abscissa: total temocillin concentration; ordinate: (a) and (d): unbound temocillin concentration (measured); (b) and (e): unbound fraction (percent of total); (c) and (f): bound temocillin concentration calculated from the difference between the respective total [for each series of replicates (c) or for each sample (f)] and unbound concentrations (for each data point). Data were used to fit by non-linear regression analysis: (i) in (a) and (d), a quadratic (second order) polynomial function; (ii) in (b) and (e), a linear function; and (iii) in (c) and (f), the SBCALD equation. (b), (c), (e) and (f) curves are shown with 95% CI; (f) dotted lines are extrapolations beyond the last experimental point up to an abscissa value of 350 mg/L. For (b) and (e), data corresponding to an abscissa value <20 mg/L were not used because the low values compared with background yielded unreliable data for foolproof curve-fitting analysis. Values of the best-fit parameters and pertinent statistical analyses: see Table 2 and Table S4. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

Table 2. Parameters of temocillin PPB based on assay of unbound and total temocillin

Parameter[a]	Healthy individuals (Group #1)[b]	General ward patients (Group #2)[b]	ICU patients (Group #3)[b]	ICU patients (Group #4)[b]
(a1) Binding in spiked plasma samples from donors not exposed to temocillin (*in vitro*)[c] (analysis of data from Figure 1c)				
R^2 (fit goodness)	0.998 ($n=126$)	0.986 ($n=42$)	0.961 ($n=63$)	0.919 ($n=49$)
K_d^d (mg/L) ± sSD[e]	26.7 ± 1.31 (A;a;α)	58.6 ± 9.91 (B;a;α)	57.6 ± 9.35 (B;a;α)	39.1 ± 8.41 (C;a;α)
(95% aCI)[f]	(24.2–29.4)	(42.0–83.7)	(42.0–79.8)	(25.6–58.7)
B_{max}^g (mg/L) ± sSD	294 ± 4.46 (A;a;α)	240 ± 15.5 (B;a;α)	179 ± 10.1 (C;a;α)	108 ± 5.90 (D;a;α)
(95% aCI)	(285–305)	(215–280)	(161–202)	(97.2–120)
Mean molar B_{max}/[albumin] ratio[h]	1.00	1.13	0.83	0.96
(a2) Binding in plasma samples from subjects having received temocillin (*in vivo*)[i] (analysis of data from Figure 1f)				
R^2 (fit goodness)	0.993 ($n=145$)	0.9650 ($n=84$)	0.950 ($n=65$)	0.792 ($n=146$)
K_d (mg/L) ± sSD	23.0 ± 2.72 (A;b)	33.9 ± 6.29 (A;b)	77.8 ± 22.0 (B;b)	84.9 ± 15.3 (B;b)
(95% aCI)	(18.5–29.2)	(23.0–49.6)	(47.4–156)	(59.5–124)
B_{max} (mg/L) ± sSD	351 ± 17.5 (A;b)	221 ± 17.8 (B;a)	231 ± 39.5 (B;a)	170 ± 14.7 (C;b)
(95% aCI)	(322–392)	(193–268)	(176–372)	(141–206)
Mean molar B_{max}/[albumin] ratio	1.15	1.13	1.17	1.37
(b) Binding in diluted spiked plasma samples from healthy donors not exposed to temocillin (*in vivo* study)[j] (analysis of data from Figure 3c)				
	Plasma from healthy individuals			
	undiluted		diluted	
[Total protein]/[albumin] (g/L)	81/48.0	57/34.8	52/30.6	40/23.5
R^2 (fit goodness)	0.998 ($n=27$)	0.997 ($n=27$)	0.996 ($n=27$)	0.992 ($n=27$)
K_d (mg/L) ± sSD	32.3 ± 3.72 (A;β)	43.8 ± 4.30 (B;β)	55.4 ± 5.82 (C;α)	106 ± 16.0 (D;β)
(95% aCI)	(26.6–44.3)	(35.9–53.8)	(44.9–69.2)	(73.89–148.5)
B_{max} (mg/L) ± sSD	312 ± 13.3 (A;α)	266 ± 9.83 (B;β)	257 ± 11.0 (B;β)	275 ± 21.7 (B;β)
(95% aCI)	(288–344)	(248–289)	(236–285)	(238–332)

Statistical analysis by row: ordinary one-way ANOVA with Tukey multiple comparison test (assuming equal standard deviations); similar results were obtained using Brown–Forsythe ANOVA or Welch's ANOVA with Dunnett's multiple comparison *post hoc* test (not assuming equal standard deviations) except for K_d in samples from individuals having received temocillin where values different from each other are flagged with different upper-case letters ($P<0.05$). In Table 2, the data in the left column always received a letter A, the next (from left to right) with a significant difference received a B, and so on. Statistical analysis by column: unpaired two-tailed *t*-test comparing *in vitro* versus *in vivo* data and values with the same lower-case letter (a or b) are not significantly different from each other. The same test was also used to compare *in vitro* data from donors in each group [#1, #2, #3 or #4] with *in vitro* data generated by spiking temocillin in undiluted or diluted plasma obtained from donors of Group #1. The comparison is based on similar albumin concentrations between plasma of donors of Group #1 versus undiluted plasma (47.42 versus 48 g/L), Group #2 and diluted plasma (33.7 versus 34.8 g/L); Group #3 and diluted plasma (34.8 versus 30.6 g/L); Group #4 and diluted plasma (17.9 versus 23.5 g/L), and values with the same lower-case Greek letter (α or β) are not significantly different.

[a]Determined by fitting Equation 1 (SBCALD) to the data using non-linear regression (least squares; no handling of potential outliers, no weighing of the data; convergence criteria: five iterations in a row changing the sum of squares by <0.0001%; each replicate ($n=3$) considered as an individual point). Raw data (unbound versus total concentrations) are those shown in Figure 1 (a–c and d–f: *in vitro* and *in vivo* studies, respectively).

[b]See Table 1 for description of Groups #1 to #4.

[c]Number of concentrations tested: 6–9; number of replicates for each concentration: 7–14; degrees of freedom: 40–138.

[d]Drug plasma protein dissociation constant.

[e]Means ± symmetrical standard deviation (sSD); representing the effect of unsystematic errors.

[f]Asymmetrical 95% CI (aCI_{95}); profile likelihood; addressing the combined effect of unsystematic errors and systematic bias, calculated using a consistency standard error and an agreement standard error.[23]

[g]Maximal binding capacity.

[h]Calculated using a molecular weight of 66.5 kDa for albumin and concentration values from Table 1.

[i]Number of data points: 55–145; no replicate (all independent) degrees of freedom: 53–143.

[j]Number of concentrations tested: 9; number of replicates for each concentration: 3; degrees of freedom: 25.

concentrations when moving successively from Group #1 to #2, #3 and #4 (Figure 1d); and (iii) linear increase of the unbound fraction as a function of the total temocillin concentration with values close to those measured *in vitro* (Figure 1e), again with similar slopes among the three groups of patients but stepwise and statistically significant increases of the offset values of the

fitted functions when moving successively from Group #1 to #2, #3 and #4 (Table S4.1.A2 and S4.1.B2). Unbound fractions reached, respectively, 4%–13%, 12%–29%, 23%–42% and 32%–52% for the four groups for 20–200 mg/L total concentrations. A high correlation was noticed between slopes and offset values from *in vitro* and *in vivo* samples [r: 1.000 and 0.979, respectively (Pearson's correlation coefficients); *P* values <0.05 and 0.01]. Lastly, *in vivo* data could be successfully used for fitting the SBCALD function (Figure 1f). Table 2.a2 shows that B_{max} parameters for Groups #1 (351 mg/L) and #4 (170 mg/L) were significantly the highest and the lowest, while the values for Groups #2 (221 mg/L) and #3 (231 mg/L) were intermediate but not significantly different from each other. Conversely, changes in K_d were more consistent, with stepwise, statistically significant increases when moving from Group #1 (23 mg/L) to #2 (34 mg/L), #3 (78 mg/L) and #4 (85 mg/L). The *in vitro* and *in vivo* data were, however, not fully superimposable; when comparing binding parameters, we observed that B_{max} was significantly more elevated for *in vivo* samples from Groups #1 and #4 only, while K_d values were significantly different for all groups and significantly more elevated for Groups #3 and #4. Nevertheless, simulation studies (Figure S3A) showed that the observed changes in B_{max} could account for the bulk of the differences in temocillin PPB seen between groups *in vivo*, while those observed for K_d had only a minor impact, yielding a global message similar (though not entirely identical) to that obtained from the analysis of the *in vitro* data. Of note, the molar ratio B_{max}/[albumin] was close to 1 in all groups for both *in vitro* and *in vivo* samples.

CRP concentration, hypoalbuminaemia and temocillin unbound fraction

No difference was evidenced in total plasma proteins, albumin and CRP concentrations between samples from donors and subjects (Table 1). There was a major and progressive decrease in total proteins in samples from Group #1 (>70 g/L) to Groups #2 and #3 (~64 g/L) and then to Group #4 (<50 g/L), essentially due to decreases in albumin concentration. CRP levels were globally elevated in patients with a statistically significant difference between Groups #2 and #3 taken together versus Group #4, but with an important overlap.

The relationship between albuminaemia, CRP concentration and temocillin unbound fraction is shown in Figure 2 for *in vivo* samples. Based on clinical scoring of hypoalbuminaemia related to mortality,[24,25] all patients in Group #4 had both severe hypoalbuminaemia (<25 g/L) and the most elevated mean CRP level (>200 mg/L; left panel). Conversely, albuminaemia remained within normal limits in all subjects of Group #1. The largest proportion of patients from Groups #2 and #3 had mild hypoalbuminaemia (<35 g/L) and moderate CRP levels (<200 mg/L), with some spread to the other two categories. There was a highly significant correlation between the decrease of albumin level and the increase of temocillin unbound fraction (right panel).

Temocillin binding in diluted plasma and pharmaceutical albumin

Figure 3 shows the results of additional experiments using spiked specimens made (i) in diluted plasma from Group #1 donors [Figure 3(a–c)] or (ii) in pharmaceutical albumin [Figure 3(d–f); see Table 2b, Table S4.2 and Figure S3B for numerical data and additional analyses]. Together with undiluted plasma, this yielded four binding curves, ordered so as to compare each of them with one of the four curves observed for donors of Groups #1, #2, #3 and #4 spiked with temocillin [compare Figure 1(a–c) and Figure 3(a–c)]. This showed total concentration-dependent saturation together with stepwise increase of unbound temocillin concentrations when moving from undiluted to increasingly diluted plasma (Figure 3a), a linear relationship between temocillin unbound fraction and total concentration (Figure 3b) and adequate fitting of data describing binding in diluted plasma to the SBCALD function (Figure 3c). Simulation studies suggested here that changes in both K_d and B_{max} parameters played a critical role (Figure S3B). Dilution of plasma to mimic the changes in albumin concentrations seen in samples from the different groups failed to fully reproduce the changes in unbound temocillin concentrations, and all values were systematically lower (Figure S4). Replacing plasma by pharmaceutical albumin resulted in still less temocillin binding and in a loss of saturation (Figure 3d). The SBCALD function could not be fitted to the data, a linear relationship being observed between total and bound concentration in this matrix all over the range of investigated concentrations (Figure 3f). A linear relationship was also maintained between unbound and total concentrations, but with slopes much lower and offset values much higher than in diluted plasma (Figure 3e versus 3b; Table S4.2.B and Table S4.3.B).

Impact of combining drugs with high PPB on temocillin PPB

Figure 4 (with additional analyses in Figure S5) shows that the effects of all competitor drugs on temocillin binding were minimal, with fluconazole causing a larger increase of unbound temocillin concentration than any of the other drugs, although it was the drug showing the lowest PPB among those tested here. This change in unbound fraction can be predicted using a modified SBCALD equation taking into account the dissociation constant of each drug from plasma proteins (Figure S6). Simulation studies also showed that this unexpected ranking observed when comparing diazepam with fluconazole was essentially due to differences in concentration ratios of the two drugs to temocillin when used at the clinically relevant plasma concentrations (Figure S7).

Discussion

Temocillin shows high PPB, calling for attention to individual and/or local unbound concentration when attempting to optimize its activity. However, the scarce and often disparate studies published so far do not provide the basic data that are needed to apprehend the clinical importance of this binding. We therefore addressed several key questions in a progressive but comprehensive fashion, including the characterization of the saturation of temocillin PPB in infected patients, the impact of inflammation and ensuing hypoalbuminaemia, the reproducibility of plasma data with pure albumin, or potential interferences of co-medications with high PPB. By using a combination of *in vitro* and *in vivo* approaches, and enrolling homogeneous classes of

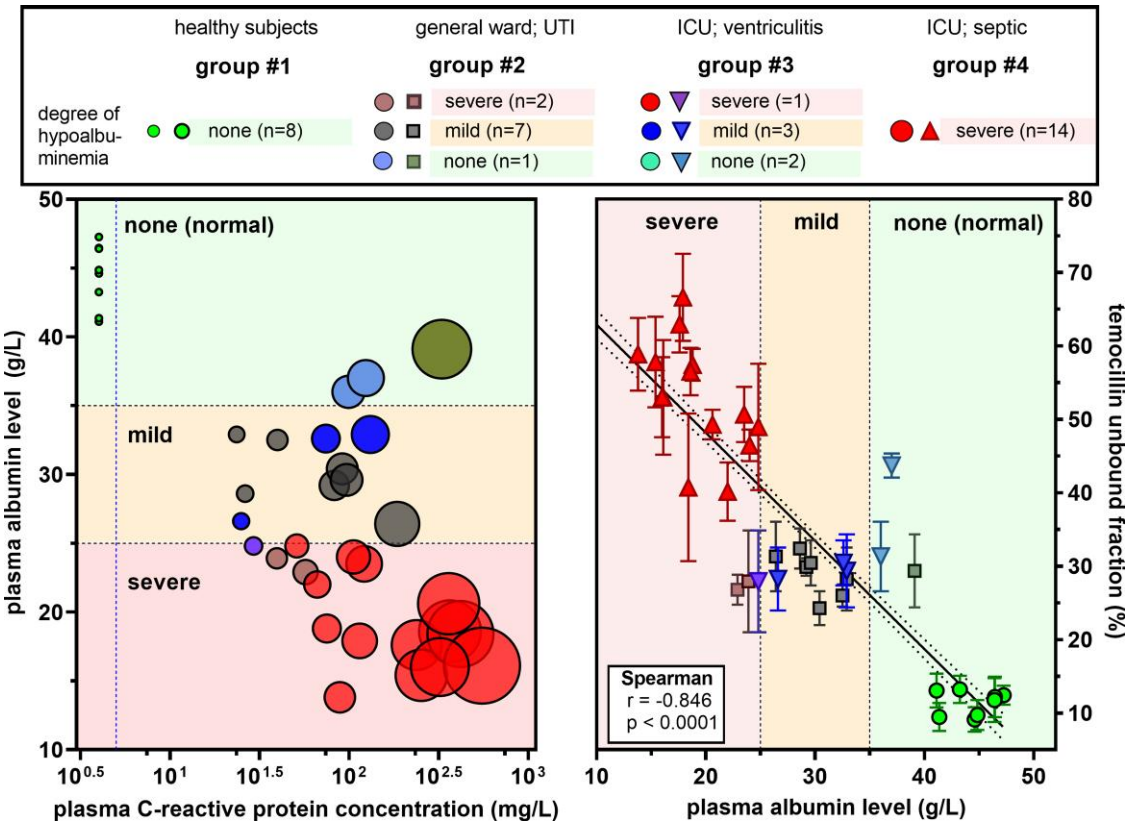

Figure 2. Albumin plasma levels versus CRP concentration and correlation between plasma albumin levels and temocillin unbound fraction in the different groups of samples. Left panel: samples from subjects of Group #1 (healthy individuals; green symbols) and from subjects of Groups #2, #3 and #4 (patients; black, blue and red symbols, respectively) were taken for routine measurement of albumin and CRP within the first 24 h of enrolment (Group #1) or of hospitalization (Groups #2, #3 and #4). Results are shown as a bubble graph where each circle represents one individual and is centred on coordinates respective to its albumin level (ordinate) and CRP concentration [abscissa; logarithmic scale (see Table 1 footnote e)] while the area of each circle is proportional to the CRP concentration of that sample. CRP and albumin concentrations were single determinations. For CRP, no sampling of healthy subjects was made; a value of 4 mg/L [international and local normal values: <5 mg/L; vertical dotted line] was attributed for graphing purposes. Right panel: correlation between albumin level (abscissa) in the same samples and the unbound temocillin fraction (ordinate). The graph shows the correlation parameters as determined using non-parametric (Spearman) statistics. Since temocillin binding was saturable, each value of unbound concentration was recalculated for a common total temocillin concentration of 200 mg/L using the SBCALD function fitted to the data of *in vivo* samples (Figure 1f) and the corresponding binding parameters of each group (see Table 2), allowing comparable unbound fractions for all individuals included in the analysis to be obtained. For both panels, each sample was also categorized according to the degree of hypoalbuminaemia into three groups (severe: <25 g/L; mild: from 25 to <35 g/L; or normal: 35 g/L or more) using the criteria proposed to clinicians to assess the mortality risks of patients experiencing hypoalbuminaemia.[24,25]

patients with respect to the severity of their infection and a cohort of healthy volunteers as control, we were able to make key observations that may pave the way for future research.

First, we showed that the binding capacity of plasma proteins for temocillin (B_{max}) is reduced in patients, in a proportion that is directly correlated with the increase in CRP and inversely correlated with the concentration of plasma albumin, two markers of inflammation and of increasing mortality risk.[24–26] This information may be of critical importance to clinicians who need to adapt dosing regimens accordingly. Decreased temocillin PPB in infected patients had been noted but was either not studied[8,9] or was considered in infected patients as a single heterogeneous group, leading to incomplete and therefore suboptimal information.[27] The design of our study, with objective stratification of patients into three cohorts with increasing severity of infection and little overlap between them, and a cohort of healthy volunteers, allowed us to mitigate this issue. Group #1 (healthy volunteers) was missing in the recent flucloxacillin PPB study,[27] for which the model also excluded critically ill patients,[28] but was important for providing needed baselines (regrettably missing for our CRP assays). A decrease in plasma albumin is a well-known feature of infection[29] and clinical categories of hypoalbuminaemia are associated with major differences in mortality.[24,25] Infection triggers inflammation, which is thought to cause albumin escape through increased capillary permeability[30] and to also shorten its half-life,[31] providing a rational, though probably incomplete, explanation for infection-associated hypoalbuminaemia.[32] Although causality remains to be documented, the available data clearly point to hypoalbuminaemia as the rational link between infection severity and decreased temocillin PPB. Yet,

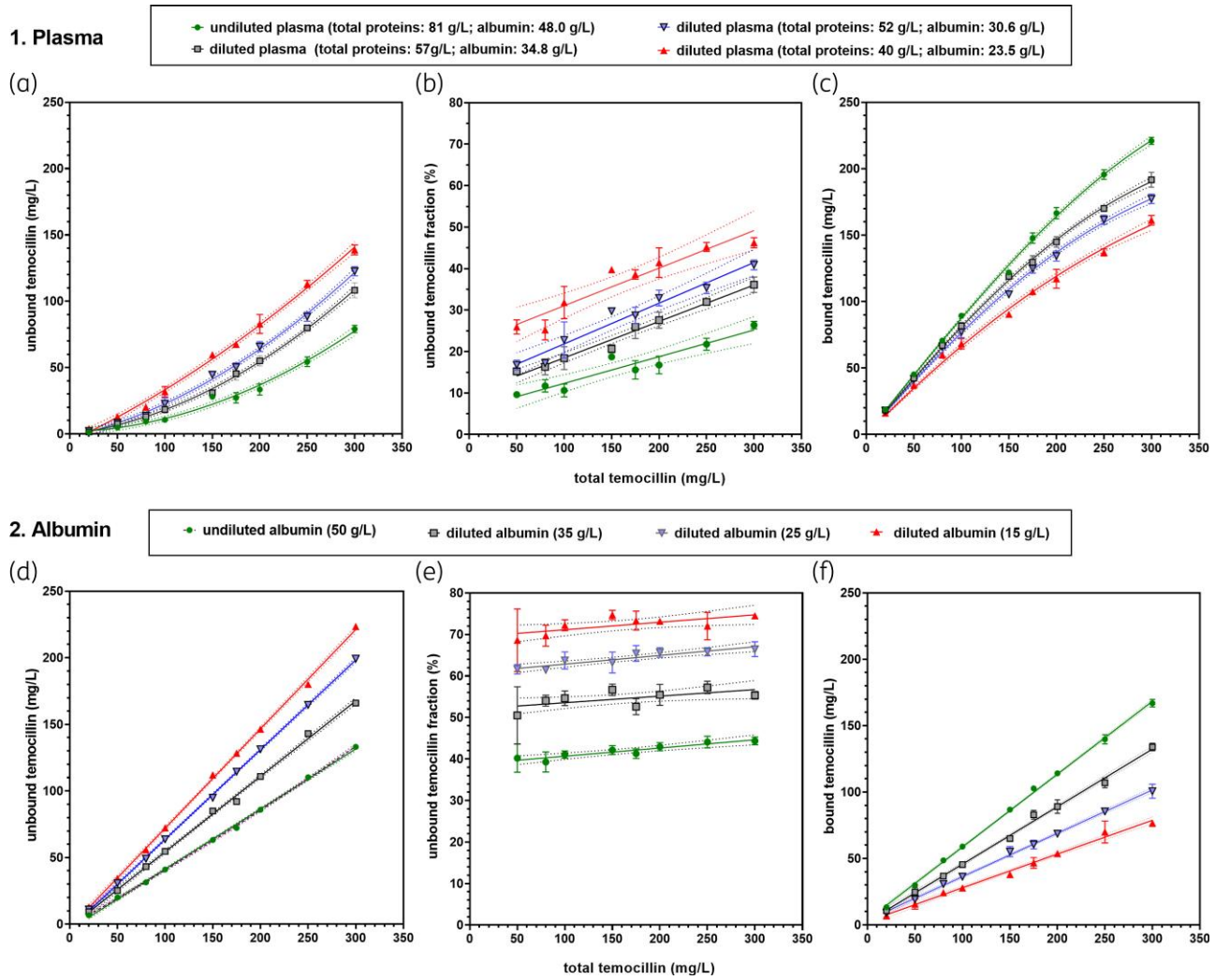

Figure 3. Binding of temocillin in diluted plasma and pure albumin. (a–f) Spiked samples from healthy subjects. (a–c) Spiking in undiluted and diluted plasma. (d–f) Spiking in undiluted and diluted pharmaceutical albumin (ALBUREX®). The dotted lines above and below each solid line show the confidence band (95% CI) of each of the corresponding fitted functions. Abscissa: total temocillin concentration; ordinate: (a) and (d): unbound temocillin concentration (measured; raw data); (b) and (e): calculated unbound temocillin fraction (percent of total); (c) and (f): bound temocillin concentration, calculated as difference between total (common to each series of replicates) and unbound (specific to each replicate) concentrations. For (b) and (e), data corresponding to an abscissa value ≤20 mg/L were not used to avoid fooling the curve-fitting analyses with intrinsically unreliable data due to low experimental readings. Data are shown as means ± SD of three (plasma) or four (albumin) replicates for each total concentration. (a) A second-order polynomial (quadratic) equation was fitted to the data. (c) The SBCALD function was fitted to the data. (f) The SBCALD function could not reliably be fitted to the data and linear functions were used for fitting. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

a maximal load of about one molecule of temocillin per molecule of albumin is maintained, as previously decribed.[33,34] Similar correlations have already been described for other antibiotics with large PPB.[35–37] Cirrhosis and ensuing hypoalbuminaemia have also been associated with decreased PPB of many highly protein-bound antibiotics.[38] Here, we also show by multilinear regression analysis that albumin decrease and CRP increase account for most of the observed effect.

It is commonly accepted that the unbound concentration prevailing at the infected site is critical for activity and therapeutic success of most antibiotics including β-lactams,[39] although clinical evidence remains scarce.[40,41] Infected sites are most often extravascular,[42] but measuring unbound concentrations in tissues or extravascular fluids is not a routine investigation in clinical practice. The unbound plasma concentration of antibiotics is often used as a surrogate for predicting activity at the infection site.[43–45] There is much evidence that this concept fully applies to β-lactams,[46–48] as these drugs do not accumulate in deep compartments.[49,50] A rapid equilibrium of the unbound concentrations of temocillin between plasma and extracellular sites is indeed expected to occur, with the bound drug acting as a reservoir favouring the diffusion of the antibiotic to the site of

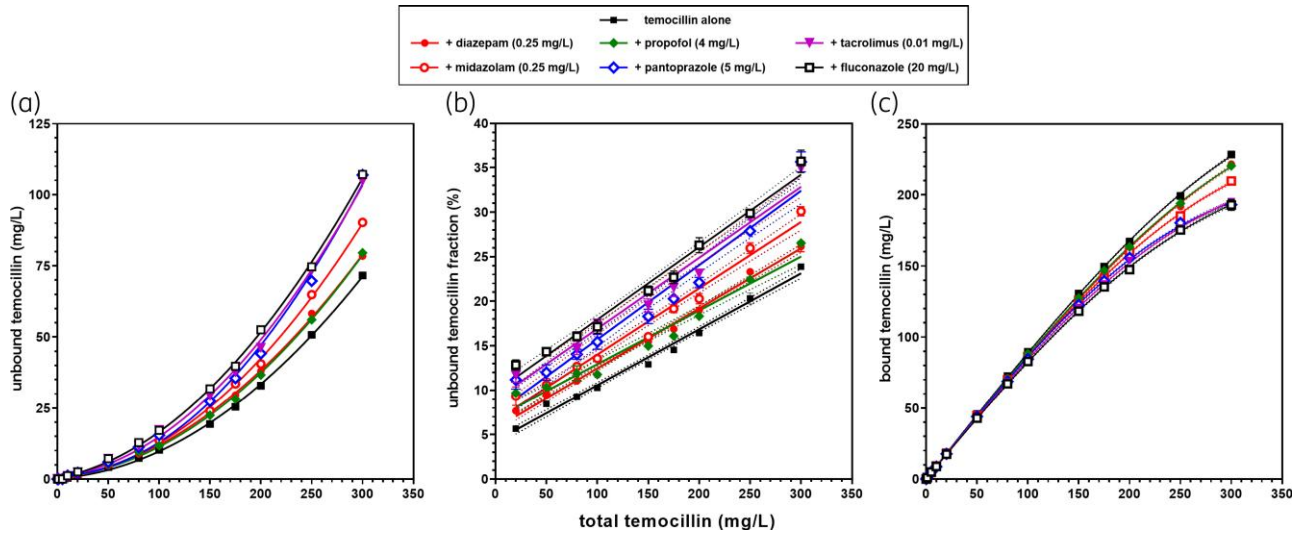

Figure 4. Impact of co-medications on the binding of temocillin to plasma proteins. (a–f) Spiked plasma samples from healthy subjects. Abscissa: total temocillin concentration; ordinate: (a) unbound temocillin concentration (measured); (b) unbound temocillin fraction (percent of total); (c) bound temocillin concentration, calculated from the difference between the respective total and unbound concentrations. Data are shown as mean ± SD (not visible if smaller than the symbols) of replicates (three for each experimental point). Data were used to fit a quadratic polynomial function (a); a linear function (b); the SBCALD function (c). The dotted lines above and below each solid line in (b) and (c) show the confidence band (95% CI) of each of the corresponding functions. For (b), data were filtered to exclude those with an abscissa value <20 mg/L from the analysis to avoid fooling the curve-fitting analyses with intrinsically unreliable data due to low experimental readings. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

infection,[51] as was originally proposed for and observed with temocillin.[4,52,53] We know that it is the time during which the unbound concentration remains above the MIC of the causative organism (%$fT_{>MIC}$)[54] that predicts β-lactam activity[55–57] and prevents the emergence of resistance.[58] However, the impact of protein binding on PK/PD is complex.[59] For example, increasing the unbound-to-bound ratio of a protein-bound drug will also cause an increase in its volume of distribution (V_d). As a consequence, its unbound concentration in the plasma will decrease. This adverse effect will be all the more important in patients with severe infections due to their hypoalbuminaemia.[35] Moreover, increasing the unbound fraction of a drug will also enhance its renal clearance.[60,61] Altogether, these factors might cause a decrease rather than an increase in %$fT_{>MIC}$. This is likely to occur in the absence of therapeutic drug monitoring (TDM)-based dose adjustments. Further documentation of the relationship between infection severity and increase in unbound temocillin concentrations will be essential, as it may help to design predictive diagnostic tools needed for optimal initiation of the therapy.[56]

Second, we show that pharmaceutical albumin binds temocillin but to a lesser extent than anticipated and, most intriguingly, fails to show saturation. Our study, however, did not provide new information on the mechanisms responsible for temocillin PPB. We noticed that the dissociation constant K_d tends to increase for *in vivo* samples from ICU patients versus volunteers or non-severely ill patients. We cannot exclude a lower affinity of albumin from ICU patients,[62] but our modelling studies (Figure S3) suggest that the overall binding of temocillin is essentially influenced by the B_{max} values. The lack of quantitative similarity of the effects exerted by a decrease in plasma albumin caused by infection versus mere plasma dilution, and the fact that replacing plasma albumin by pharmaceutical albumin yielded a much lower and non-saturable binding, suggest a role for other proteins or plasma constituents. It is also possible that diluting plasma with 0.9% NaCl introduced changes in albumin-binding properties. Moreover, we cannot rule out that the pharmaceutical albumin used here did not maintain all key properties critical for drug binding.[63–67] In this context, differences between brands could explain why clinical trials examining the improvements in organ function brought by albumin supplementation in severely ill patients yielded confusing results, leading to variable clinical recommendations.[68–71]

Third, we document that the co-administered drugs with high PPB only cause marginal increases in unbound temocillin concentration if from a class for which activity develops at lower molar concentrations than temocillin. Conversely, drugs with low PPB may trigger interferences if their active concentration is similar to that of temocillin. This can probably not be ascribed to a difference in the binding site between temocillin and competitors, since benzodiazepines or propofol bind to the drug binding site I of albumin, but pantoprazole, as well as some β-lactams, to its drug binding site II.[72] The simplest alternative explanation is that we tested these drugs widely used in ICU at the concentrations at which they exert their pharmacological effect, which are, on a molar basis, much lower than those of temocillin. Conversely, our data with fluconazole may warn clinicians about unanticipated effects of drugs with low protein binding if these require therapeutic plasma concentrations to reach values close to those of temocillin.

Beside its direct impact for the better appraisal of the parameters affecting protein binding and its consequence in the

clinics, this work also offered us the opportunity to reassess the H–Le equation and its applicability in the context of ligand depletion often seen in pharmacological situations. Many studies, including recent ones,[4,14,27,28,60] use the H–Le equation forgetting that it was developed for ligand–receptor studies run under conditions of minimal binding, but not for conditions of high ligand concentrations causing substrate depletion. This condition invalidates the H–Le equation and all similar functions,[73,74] and is specifically not recommended if the unbound ligand concentration is <90% of its total concentration,[23] which was the case here. Moreover, bound drug concentrations (i.e. the dependent variable in H–Le) were not measured. In general, these are calculated as the difference between total and unbound concentrations. However, the H–Le would become implicit in these conditions, requiring both specific calculation methods and providing initial values for the parameters to be determined by curve-fitting analysis.[23] Based on previous work addressing the ligand depletion issue in pharmacological studies,[22] and on previous studies with ceftriaxone[13] and temocillin,[75] we developed an explicit equation that takes ligand depletion into account and uses the total temocillin concentration as independent variable. Of note, both the total and unbound concentrations are experimentally measured in clinical samples (and thus both accompanied by an experimental error), while total concentration is known in *in vitro* samples. This may possibly contribute an explanation for the higher variability observed in binding parameters calculated for *in vivo* versus *in vitro* samples.

In conclusion, our data show that PPB of temocillin is lower in infected patients than in healthy subjects, the difference being correlated with the degree of severity of their infection and commensurate decrease in plasma protein levels. In a broader context, it suggests the importance of studying drug PPB in the target patient populations. Because *in vivo* temocillin PPB could not be fully reproduced *in vitro*, our study highlights the importance of monitoring the unbound concentration *in vivo*. We also show that studying the potential interactions of any drug with temocillin PPB should focus on therapeutically pertinent concentrations in order to ensure clinical relevance. Our data also warn against indiscriminate use of pure albumin or plasma from healthy volunteers in the study of PPB of drugs.

In a broader context, this comprehensive work of temocillin PPB may be considered as a normative study, which not only provides a pharmacological model to estimate bound concentrations based on total concentrations and binding parameters, but also highlights for the clinician some key parameters to take into account to establish rational dosing for highly protein-bound drugs, as well as to select optimal conditions for treatment in the clinic, especially when TDM is performed.[76]

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Transparency declarations

P.N.P. is an employee of the Université catholique de Louvain. X.W. and P.-F.L. are employees of the Cliniques universitaires Saint-Luc, and S.V. and L.G. of the AZ Delta Ziekenhuis. F.V.B. is Research Director of the Fonds de la Recherche Scientifique (F.R.S.-FNRS). P.M.T. is an Emeritus Professor and was unpaid. The laboratories and/or clinical units of F.V.B., P.M.T., A.E.M., M.Z. and P.-F.L. have received research supporting grants and/or honoraria from various industries for research work and/or presentations unrelated to the topic of the present paper. All other authors declare no conflicts of interest.

Supplementary data

Supplementary Materials and methods, Tables S1 to S4, Figures S1 to S7 and the Appendix are available as Supplementary data at JAC Online.

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Supplementary data

Materials and Methods

Drugs and Reagents (others than temocicllin)

Human albumin (ALBUREX® 5% for injection; >96% human albumin) was from CSL Behring GmbH (Marburg, Germany), apyrogenic 0.9% NaCl, from Baxter Healthcare SA (Zurich, Switzerland), ticarcillin disodium (internal standard), from Sigma-Aldrich Corp. (St. Louis, MO), HPLC-MS grade methanol and acetonitrile, from J.T. Baker (Deventer, The Netherlands), formic acid and ammonium acetate, from Merck KGaA (Darmstadt, Germany).

Biochemical assays

Validated automated methods were used for assay of total proteins (Biuret),[1] albumin (bromocresol green dye)[2] or C-reactive protein (CRP; immunoturbidimetry),[3] respectively (Cobaz® analyser 8000 series, Roche Diagnostics, Rotkreuz, Switzerland).

Additional control studies or assessments for temocillin assay

Samples for the study of matrix dilution effects

For experiments looking at the effect of matrix dilution, plasma pooled from healthy subjects or albumin were diluted with NaCl 0.9% in order to obtain total protein and albumin concentrations close to those observed in samples from donors of groups #2, #3, and #4, before spiking TMO.

Evaluation of temocillin binding to surfaces of the centrifugation devices

Binding of TMO to the surface of the Amicon® cartridges (cut-off: 30 kDa) used for separation of the unbound drug was assessed by measuring the concentration of temocillin in the lower chamber of the device, using samples spiked in NaCl 0.9% and (a) added direcly to the lower chamber of the device (no filtration or centrifugation) or (b) to the upper chamber of the device and centrifuged as plasma samples (5 minutes at 11,000 *g*) in order to recover the unbound temocillin in the lower part of the device (note that all fluid passed into the lower chamber during centrifugation since there was no plasma).

Influence of the temperature of centrifugation on temocillin recovery from the Amicon® ultrafiltration devices.

Previous studies suggested that the temperature at which samples were centrifuged in the ultrafiltration device may influence the recovery of the unbound molecule.[4] We therefore looked for an impact on temocillin using samples from temocillin-naïve individuals belonging to the groups contributing to this study and spiked with temocillin total concentrations known to collectively generate unbound concentrations within a span (2-140 mg/L) corresponding roughly to that seen in our study. All samples were splitted in two aliquots that were processed according to the same general procedure except than one of them was centrifuged during 5

minutes at 11,000 *g* at 25°C (40% of the volume ultrafiltrated), as done in all previous studies having measured temocillin unbound concentrations,[5-8] and the other, at 37°C.

Impact of dugs with high PPB, and of tacrolimus and fluconazole on TMO-PPB

We looked for a change in the unbound fraction of temocillin in healthy volunteers' plasma samples spiked with a fixed concentration of competing drug. In a a first approach, TMO was added at two selected total concentrations (50 and 150 mg/L) alone or in the presence the competing drug at a concentration corresponding to what is observed in the serum of patients given the drug for therapeutic purposes (diazepam, 0.25 mg/L; midazolam, 0.25 mg/L; propofol, 4 mg/L; pantoprazole, 5 mg/L; tacrolimus, 0.01 mg/L; fluconazole, 20 mg/L (see[9] and the specific regulatory data related to approved indications). In a second approach, the same procedure was applied but using a whole range of temocillin concentrations. In all samples, temocillin unbound concentration was measured after 30 minutes incubation at 37°C. Increases in temocillin unbound concentrations were interpreted as evidence of displacement by the added comedication. In addition, bound concentrations were calculated using the SCBALD equation.

Data distribution, statistical and curve-fitting analyses.

All data sets (and their transformants if suitable) were checked for distribution normality before any analysis, using a panel of 4 tests (Anderson-Darling, D'Agostino-Pearson omnibus K2, Shapiro-Wilk (using the method of Royston), and Kolmogorov-Smirnov). In situations where the number of individual data was too low (as was often the case with data from group #3), the two former tests could not be made and were discounted. Normality (negative answer to the test) was assumed if one test was negative. Non-nomal distributions, or distributions for which normality was uncertain, were analyzed using non-parameteric statistics.

Curve-fitting analyses were made using two appoaches. First, we aimed at finding an equation best fitted to the raw data, using a polynomial function and the Akaike' method rationally choosen between potential candidates (most often distinguishing between linear and quadratic functions). Second, a mechanistic model was selected using theoretical considerations and in-depth inspection of the data, and the corresponding equation was best-fitted to the data by simple non-linear regression analysis and search for best fitting values of its parameters. This led us to select the SBCALD equation (derived from the Hill-Langmuir equation but taking into acount the important ligand depletion observed in most antibiotic-PPB studies) and, therefore to obtain best fitted values of its K_d and B_{max} parameters (drug-plasma protein dissociation constant and maximal binding capacity, respectively, all expressed in mg of drug/L for convenience). The quality of the curve-fitting analyses was documented by computing the "goodness of fit" (R^2; reported for each analysis) and the standard deviation of the residuals (sy.x; see comment in Tables) as well as by visual inspection of the data and of the proposed fitted function. Additional controls and quality assessments were made by inspecting "quantile-quantile" (QQ) and "calculated vs observed" data plots. Quality controls for other specific studies and reported in the captions of the correspondig Figures. Prism (versions 9.2 and 9.4; Graphpad Software, San Diego, CA) was used throughout.

Results

Tables

Table S1: Demographic, biological, and medical data of the study populations

Parameter	Study populations			
	healthy individuals	Patients[1]		
		in general ward	in ICU	
	group #1	group #2	group #3	group #4
Donors: In-vitro study (spiking of plasma samples from donors unexposed to temocillin) [2]				
General				
number (n)	14	7	9	7
age (years) (median-range)	28 [20-38]	82 [68-90]	70 [55-78]	71 [54-79]
Co-medication(s) (number of donors treated / number of individuals in group) [3]				
Diazepam	0	**3/7**	0	0
Midazolam	0	0	0	0
Propofol	0	0	**2/9**	**6/7**
Pantoprazole	0	**2/7**	0	**4/7**
Tacrolimus	0	0	0	**2/7**
Fluconazole	0	0	0	**1/7**

Subjects: In-vivo study (plasma samples obtained from subjects having received temocillin)				
General				
number (n)	14	12	7	14
median age (years) [range]	27 [23-55]	75 [49-90]	58 [54-60]	56 [36-80]
weight (kg ± SD)	81.9 ± 10.9	88.7 ± 18.6	76.67± 7.99	73.2 ± 15.3
body mass index (kg/m^2 ± SD)	24.4 ± 2.9	30.3 ± 5.64	27.4 ± 3.55	25.6 ± 4.46
Median GFR [4] (mL/min) ± sSD	1182 ± 24.6	61.3 ± 19.1	199 ± 33.5	43.9 ± 34.6
Plasma enzymes (units/L; nl: normal local values) [5]				
GGT (nl. 9-48)	not done	53.0 ± 37.5	38.5 ± 8.9	99.3 ± 60.5
AAT (nl: 29-33)	30.1 ± 9.2	30.9 ± 25.4	32.8 ± 16.1	43.1 ± 32.3
AST (nl: 5-40)	23.8 ± 4.5	29.0 ± 11.1	23.7 ± 7.99	53.26 ± 47.0
Infection (type, main pathogen isolated from infection site and from haemoculture				
diagnosis [6] (no. in population)	not applicable	cUTI 12/12	suspected ventriculitis 7/7	UTI (1/14) IAI (10/14) LRTI (3/14)
Pathogen isolated (no. positive patients)	not applicable	*E. coli* (8) *K. pneumoniae* (3) *S. epidermidis* (1)	*E. coli* (1) *K. pneumoniae* (3) *E. cloacae* (2) *E. aerogenes* (1)	*E. coli* (8) *K. pneumoniae* (4) *E. cloacae* (2)
Positive haemoculture(s) (no. pf patients) and pathogen(s) isolated (no. of patients)	not applicable	0	1 *K. pneumoniae* (1)	9 *E. coli* (4) *K. pneumoniae* (4) *P. mirabilis* (1)
Marker of inflammation: see Table 1 (main manuscript)				

Global disease severity				
SOFA score [7]	not done	not done	3 ± 1	9.93 ± 3.32
APACHE II score [8]	not done	not done	16.7 ± 3.85	20.4 ± 6.07
Temocillin exposure or treatment before sampling				
daily dose (g)	2	4	6 [9]	6 [9]
mode of administration and schedule	40 min infusion (single dose)	30 min infusion Q12h	Initial loading dose (2g in 30 min) followed by continuous infusion	Initial loading dose (2 g in 20 min) followed by continuous infusion
exposure length (± SD)	12 h [10]	4 ± 1 days [11]	3.5 ± 1.0 days [12]	6.5 ± 2.4 days [12]
Comedication(s) (number of individuals in population) [6]				
diazepam	0	2/12	0	0
midazolam	0	0	2/7	2/14
propofol	0	0	2/7	5/14
pantoprazole	0	2/12	2/7	5/14
tacrolimus	0	0	0	4/14
fluconazole	0	0	0	0

[1] Patients with documented UTI hospitalized in the Department of Urology of the *AZ Delta Ziekenhuis* (**Group #2**); patients hospitalized in ICU at *Cliniques universitaires St-Luc* with suspected/documented ventricular infection (**Group #3**) or with sepsis/septic shock (**Group #4**).

[2] plasma samples from individuals untreated with TMO and used for *in-vitro* spiking with TMO followed by measurement of unbound TMO

[3] limited to the co-medications used to investigate their impact on TMO-PPB in this study

[4] calculated glomerular filtration rate using serum creatinine data and the Cockcroft-Gault equation.

[5] abbreviations: GGT: γ-glutamyl-transferase; AAT: alanyl-aminotransferase; AST: aspartate aminotransferase

[6] abbreviations and comments: UTI: Urinary Tract Infections; cUTI: complicated Urinary Tract Infections, IAI: Intra-Abdominal Infections (all subjected to surgery); LRTI: Lower Respiratory Tract Infections (pneumonia and severe infectious broncho-pneumopathy)

7 SOFA: Sequential Organ Failure Assessment score[10]

8 APACHE II: Acute Physiology And Chronic Health Evaluation II score[11]

9 not including the initial loading dose

1 0healthy volunteers were sampled at predefined times during the 12h following a single dose administration

11 patients were sampled at predefined times during the 12h interval separating two administrations after the number of days of treatment as indicated

12 first sample obtained 30 min after infusion of the loading dose, second sample during the continuous infusion period, and 5 additional samples obtained at predefined times over 12h following treatment discontinuation

Table S2: Best fitted values of the K_d and B_{max} parameters calculated using the SBCALD function fitted to the data of each group using the data of Figure 1 panel C (centrifugation step in the ultrafiltration device at 25°C) or after correction to mimic the performance of the centrifugation step at 37°C; see equation in Figure S2-B). The figure below the Table shows the results of the application of two diagnostic tools for testing the quality of the submitted data and the correct performance of the non-linear regression analysis.

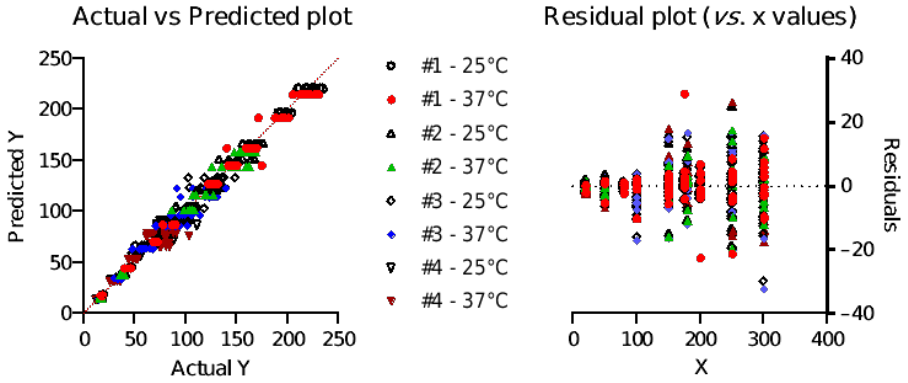
This table is a copy of Table 2-A1 (main document) complemented with (i) values of the K_d and B_{max} parameters obtained after application of the correcting factor to original data obtained with a temperature of centrifugation of 25°C to mimic the values that would have been obtained if centrifugation would have been carried out at 37°C.

Parameter [1]	**healthy individuals**	**patients**		
		in general ward	**in ICU**	
	(group #1) [2]	**(group #2)** [2]	**(group #3)** [2]	**(group #4)** [2]
A1. Binding in spiked plasma samples from donors not exposed to temocillin (*in vitro*)[3] (analysis of data from Figure 1C)				
original data * R^2 (fit goodness) / n	0.998 / n=126	0.986 / n = 42	0.961 / n = 63	0.919 / n= 49
*corrected data ** R^2 (fit goodness)*	0.993	0.983	0.949	0.875
original data * K_d [4] (mg/L) ± sSD [5] (95% aCI) [6]	26.7 ± 1.31 [**A**] (24.2 to 29.4)	58.6 ± 9.91 [**B**] (42.0 to 83.7)	57.6 ± 9.35 [**B**] (42.0 to 79.8)	39.1 ± 8.41 [**C**] (25.6 to 108.7)
*corrected data ** K_d [4] *(mg/L) ± sSD* [5] *(95% aCI)* [6]	*28.9 ± 2.36 **[A]** 24.7 to 30.6*	*58.6 ± 10.4 **[B]** 41.3 to 85.2*	*56.3 ± 9.78 **[B]** 39.2 to78.5*	*32.9 ± 8.63 [**A**] 197 to 53.0*
original data B_{max} [7] (mg/L) ± sSD (95% aCI)	294 ± 4.46 [**A**] (285 to 305)	240 ± 15.5 [**B**] (215 to 280)	179 ± 10.1 [**C**] (161 to 202)	108 ± 5.90 [**D**] (97.2 to 120)
corrected data B_{max} [7] (mg/L) ± sSD (95% aCI)	*288 ± 7.89 **[A]** 234 to 306*	*225 ± 14.8 **[B]** 200-263*	*162 ± 9.49 **[C]** 146 to 184*	*90.7 ± 8.63 **[D]** 31 to 103*

* see Table 2 (centrifugation was at 25°C).

** corrected to assume centrifugation at 37°C (see Figure S2-B for the correcting function: *corrected value = (observed value ∗ 1.06) + 0.466*

Statistical analysis: Analysis by row: Ordinary one way ANOVA with Tukey multiple comparisons test (assuming equal standard deviations): similar results were obtained using Brown-Forsythe ANOVA or Welsch's ANOVA with and Dunnett's multiple comparison post-test (not assuming equal standard deviations) except for K_d in samples from individuals having received temocillin where values different from each other are flagged with different bold italic upper case letters ($p < 0.05$). In this Table, the data in the left column received always a letter **A**, the next (from left to right) with a significant difference received a **B**, and so on.



The corrected data reinforces the conclusion that variations in K_d among groups are not biologically significant, and that the decrease in B_{max} from group #1 to group #4 is significant. Additional details and figures are provided as Supplementary Figure S2B-C.

Table S3: Interactions of selected drugs with variable with PPB on temocillin-PPB as measured by increase of its unbound concentration: main drug properties and conditions of assay.

		Competing drug				Temocillin concentration (mg/L)		
Name	**Binding site to albumin**	**reported binding (plasma or serum)**		**Concentration tested [a]**		**50**	**150**	**300**
		%	**K_d [b]**	**mg/L**	**molar**	**TMO / competing drug molar ratio [d]**		
Diazepam	II[12]	98 [13]	5.88×10^{-7} [14]	0.5 [9]	1.76×10^{-6}	68.7	206.06	412.11
Midazolam	II[12]	> 96 [15]		0.5 [9]	1.53×10^{-6}	78.6	235.79	471.58
Propofol	II[16]	> 96 [17]	6.6 to 12.2 $\times 10^{-6}$ [17]	4 [9]	2.44×10^{-5}	5.38	16.13	32.26
Pantoprazole	I[18]	96 [19]	1.3 to 3.8 $\times 10^{-4}$ [20,21]	5 [9]	$1.3.0 \times 10^{-5}$	9.25	27.75	55.49
Tacrolimus	nd[e]	73 [22]	4.47×10^{-6} [23] [c]	0.01 [9]	1.24×10^{-8}	9698	29095	58191
Fluconazole	nd[e]	11-12 [24]	1×10^{-4} [25] to 6×10^{-4} (albumin) [26]	20 [9]	6.53×10^{-5}	1.85	5.56	11.18

Assay conditions: measurement of unbound and total TMO concentrations in plasma samples from healthy volunteers spiked with TMO alone at final concentrations spanning from 10 to 300 mg/L (2.41×10^{-6} to $7.32.x\ 10^{-4}$ M; 13 different concentrations, each tested in triplicate) or with TMO plus one of the competing drug

[a] final concentration of the competing drug after addition to plasma spiked with TMO, and corresponding to the reported plasma concentration typically observed in patients receiving therapeutic doses of the drug (cross-checked with optimal target plasma concentrations recommended when undertaking therapeutic drug monitoring).

[b] reported dissociation constant of the competing drug when tested for PPB (see reference).

[c] value reported for specific binding of tacrolimus to plasma protein, knowing that most of the administered tacrolimus binds to cellular constituents and red blood cells

[d] molar concentration of TMO divided by molar concentration of competing drug.

[e] binding site unknown (not determined)

Table S4: Regression parameters and statistical analyses of polynomial (not model-committed) functions[a] fitted to the **raw data** (measured **TMO unbound concentration**) and calculated **TMO unbound fraction** [unbound concentration / total concentration ∗ 100], both as a function of the **total TMO concentration.** The Table shows the regression parameters of the following equations: **Y = B0 + B1∗X** and **Y = B0 + B1∗ X + B2∗X²** (1st order (linear) and 2d order (quadratic) function, respectively).

1. TMO binding to plasma from different populations (groups #1 to #4; in-vitro and in-vivo)				
Parameters [c]	**Study populations (groups)** [b]			
	healthy individuals	**patients**		
		in general ward	**in ICU**	
Name [d] →	**group #1**	**group #2**	**group #3**	**group #4**
1.A.: Raw data (Figure 1 panels A and D; fitting of a 2d order (quadratic) polynomial) [a]				
1.A.1. In-vitro (spiked samples from individuals not exposed to temocillin; data from Figure 1 panel A)				
no. (data points) [e]	126 (9 ∗ 14)	42 (6 ∗ 7)	56 (7 ∗ 8)	49 (7 ∗ 7)
R^2 (fit goodness) [f]	0.984	0.980	0.980	0.988
B0 ± sSD [g] (95% aCI)	0.172 ± 0.865 5.83 x 10^{-3} to 3.43	0.309 ± 2.71 -5.18 to 5.80	0.808 ± 2.94 -5.07 to 6.69	0.525 ± 3.19 -5.90 to 6.95
B1 ± sSD (95% aCI)	(-3.00 ± 12.8) x 10^{-3} **[A]** (-28.3 to 22.3) x 10^{-3}	0.158 ± 0.0423 **[B]** 0.0721 to 0.243	0.220 ± 0.0445**[C]** 0.131 to 0.309	0.312 ± 0.0483 **[D]** 0.215 to 0.409
B2 ± sSD (95% aCI)	(8.60 ± 0.393) x 10^{-4} **[A]** (7.82 to 9.38) x 10^{-4}	(9.46 ± 1.31) x 10^{-4} **[B]** (6.80 to 12.1) x 10^{-4}	(1.13 ± 0.136) x 10^{-3} **[C]** (8.53 to 14.0) x 10^{-4}	(1.33 ± 0.147) x 10^{-3}**[D]** (1.03 to 1.62) x 10^{-3}

1.A.2. In-vivo (samples from individuals having received temocillin) (Figure 1 panel D)				
no. of data points	145	83	55	136
R^2 (goodness of fit)	0.858	0.874	0.909	0.927
B0 ± sSD (95% aCI)	0.0541 ± 1.24 -1.90 to 2.99	1.66 ± 3.35 -5.00 to 8.32	-1.30 ± 3.48 -8.27 to 5.68	-8.10 ± 4.36 -16.7 to 0.522
B1 ± sSD (95% aCI)	0.0173 ± 0.0213 [**A**] -0.0248 to 0.0594	0.0612 ± 0.0620 [**B**] -0.0622 to 0.185	0.260 ± 0.0674 [**C**] 0.125 to 0.395	0.449 ± 0.0570 [**D**] 0.336 to 0.561
B2 ± sSD (95% aCI)	(5.17 ± 0.727) x 10^{-4} [**A**] (3.73 to 6.31) x 10^{-4}	(1.12 ± 0.249) x 10^{-3} [**D**] (0.623 to 1.61) x 10^{-3}	(7.60 ± 3.08) x 10^{-4} [**C**] (1.43 to 13.8) x 10^{-4}	(6.14 ± 1.69) x 10^{-4} [**B**] (2.81 to 9.48) x 10^{-4}

Analysis comparing the functions fitted to the in-vitro and in-vivo raw data (1.A.2 vs. 1.A.1)
(correlation between all values of B1 and B2 parameters in vitro with all values of the corresponding parameters in-vivo)

- B1 parameter (linear component of the response):
  Spearman's (non parametric): r = 1.00, p=0.0833 (2-tailed; trend) and <0.0001 (1-tailed)
- B2 parameter: no correlation (all p-values > 0.05)
- The B0 parameter was considered as non-pertinent.

1.B. Unbound fraction: fitting of a 1st order (linear) polynomial function [a]				
1.B.1. In vitro (spiked samples from individuals not exposed to temocillin; data: Figure 1 panel B)				
no. of data points	126	42	53	43
R^2 (goodness of fit)	0.951	0.863	0.774	0.877 [8]
B0 ± sSD (95% aCI)	3.58 ± 0.226 [**A**] 3.13 to 4.03	15.7 ± 1.13 [**B**] 13.4 to 18.3	22.8 ±1.39 [**C**] 20.0 to 25.6	32.4 ± 1.27 [**D**] 29.8 to 34.1
B1 ± sSD (95% aCI)	0.0724 ± 0.00139 [**A**] 0.0695 to 0.0751	0.0955 ± 0.00601 [**A**] 0.00833 to 0.00108	0.110 ± 0.00788 [**B**] 0.0940 to 0.126	0.129 ± 0.00715 [**D**] 0.114 to 0.144

1.B.2. In-vivo (samples from individuals having received temocillin; data: Figure 1 panel E)				
no. of data points	145	82	64	134
R^2 (goodness of fit)	0.550	0.530	0.457	0.429
B0 ± sSD (95% aCI)	3.28 ± 0.149 [**A**] 2.45 to 4.10	9.63 ± 1.2 [**B**] 7.11 to 12.1	21.0 ± 1.837 [**C**] 17.4 to 24.7	29.3 ± 1.83 [**D**] 25.7 to 32.9
B1 ± sSD (95% aCI)	$(4.48 \pm 0.339) \times 10^{-2}$ [**A**] $(3.81 \text{ to } 5.15) \times 10^{-2}$	$(9.80 \pm 0.104) \times 10^{-2}$ [**B**] 0.0771 to 0.118	0.105 ± 0.0164 [**C**] 0.0737 to 0.137	0.114 ± 0.0120 [**D**] 0.0949 to 0.141

Analysis comparing the functions fitted to the in-vitro and in-vivo free fraction data (1.B.2. vs. 1.B.1)

- B1 parameters: Spearman's (non-parametric): r=1.00; p=0.0833 (2-tailed; trend) and 0.0417 (1-tailed)

2. Effect of plasma dilution on TMO binding (in-vitro)

Parameter	**Plasma** [h]			
	undiluted	**diluted** [i]		
	Dilution concentration (g/L): total proteins / albumin			
	81.0 / 48.0	**57.0 / 34.8**	**52 / 30.6**	**40 / 23.5**
Name →	**plasma undil.#1**	**plasma dil.#2**	**plasma dil.#3**	**plasma dil.#4**
*2.A. **Raw data** (in vitro only) : fitting of a 2[d] order (quadratic) polynomial[a]; data from Figure 3 panel A; n=27 for each dilution*				
R^2 (goodness of fit)	0.982	0.993	0.994	0.953
B0 ± sSD (95% aCI)	1.48 ± 2.01 2.66 to 5.03	-0.311 ± 1.77 -3.66 to 3.84	-2.12 ± 1.85 -5.94 to 1.70	-5.50 ± 2.86 -10.2 to -0.844
B1 ± sSD (95% aCI)	0.0246 ± 0.0297 [**A**] -0.0366 to 0.00858	0.0950 ± 0.0261 [**D**] 0.0410 to 0.149	0.166 ± 0.0274 [**A**] 0.0.109 to 0.222	0.398 ± 0.0534 [**B**] 0.269 to 0.407

B2 ± sSD (95% aCI)	(7.64 ± 0.911) x 10^{-4} [**B**] (5.76 to 9.52) x 10^{-4}	(8.95 ± 0.803) x10^{-4} [**D**] (7.30 to 10.6) x 10^{-4}	(8.24 ± 0.840) x 10^{-4} [**C**] (6.51 to 9.97) x 10^{-4}	(5.00 ± 1.03) x 10^{-4} [**A**] (2.89 to -7.12) x 10^{-4}

Analyses comparing responses to dilution (2.A.) to responses to population groups (1.A.1.; see rationale in note [h])

- B1 parameter: Pearson's (parametric): r=0.964; p=0.0366 (2-tailed)
- B2 parameter: Pearson's (parametric) r=0.991; p=0.0093 (2 tailed)
- altogether: Pearson's (parametric): r=0.995; p < 0.001 (2 tailed)

(the parameter B0 was considered as non-pertinent)

2.B. Unbound fraction *(in vitro only) : fitting of a 1st order (linear) polynomial[a]; data from Figure 3 panel B, n= 24 (each dilution)*

R^2 (goodness of fit)	0.865	0.938	0.922	0.854
B0 ± sSD (95% aCI)	5.89 ± 0.985 [**A**] [d] 3.85 to 8.93	9.69 ± 0.878[**B**] 7.87 to 11.5	12.0 ± 1.11[**C**] 9.73 to 14.3	22.0 ± 1.40[**D**] 19.0 to 25.0
B1 ± sSD (95% aCI)	(6.43 ± 0.542) x 10^{-2} [**A**] (5.31 to 7.56) x 10^{-2}	(8.78 ± 0.483) x 10^{-2} [**B**] (7.78 to 9.78) x 10^{-2}	(9.81 ± 0.608) x 10^{-2} [**B**] (8.55 to 11) x 10-**2**1	(9.07 ± 0.800) x 10^{-2} [**B]** (7.41 to 10) x 10^{-2}

Analyses comparing responses to dilution (2.B.) to responses to population groups (1.B.1.; see rationale in note [h])
(only the parameter B0 was considered pertinent; only the 1st order(linear) was included in the analysis)

- Spearman's (non-parametric): r=1.000; p < 0.0833 ((2 tailed, trend) – p=0.0417 (1 tailed))

3. TMO binding in pharmaceutical albumin (ALBUREX®)

Parameters	**pharmaceutical albumin (g/L)** [k]			
	50	**35**	**25**	**15**
Name →	**pharm-alb #1**	**pharm-alb #2**	**pharm-alb #3**	**pharm-alb #4**

3.A. Raw data *(in vitro only) – fitting to a 1st order (linear) polynomial ; data from Figure 3 panel D; n=36 for each albumin concentration*

R^2 (goodness of fit)	0.997	0.995	0.995	0.998
B0 ± sSD (95% aCI)	-3.78 ± 0.761 -5.33 to -2.24	-2.38 ± 1.04 -4.40 to 0.0584	-3.65 ± 0.858 -5.47 to -1.82	-2.53 ± 1.22 -5.01 to 0.0493
B1 ± sSD (95% aCI)	0.451 ± 0.00480 [**A**] 0.442 to 0.450	0.567 ± 0.00666 [**B**] 0.553 to 0.580	0.673 ± 0.00523 [**C**] 0.663 to 0.684	0.741 ± 0.00712 [**D**] 0.731 to 0.760
No correlation analysis made because the function fitting this set of data is 1st order (linear) and the corresponding matches (spiking in samples from population groups (1.A.1) or in samples of diluted plasma (2.A.) are 2[d] order (quadratic). (See note [j] for exclusion of the 2[d] order (quadratic) polynomial that could have been fitted to a unique set of data here).				
3.B. Unbound fraction (in vitro only) : *fitting of a 1st order (linear) polynomial[a]; data from Figure 3 panel E; n=33 for each albumin concentration*				
R^2 (goodness of fit)	0.487	0.155	0.528	0.174
B0 ± sSD (95% aCI)	38.7 ± 0.672 [**A**] 37.3 to 40.1	52.0 ± 1.23 [**B**] 49.5 to 54.0	60.8 ± 0.657 [**C**] 59.4 to 62.1	69.4 ± 1.30 [**D**] 66.7 to 72.0
B1 ± sSD (95% aCI)	1.97 ± 0.370) x 10^{-2} [**B**] (1.22 to 2.73) x 10^{-2}	(1.59 ± 0.677) x 10^{-2} [**A**] (0.204 to 2.97) w 10^{-2}	(2.09 ± 0.361) x 10^{-2} [**B**] (1.35 to 2.83) x 10^{-2}	(1.79 ± 0.714) x 10^{-2} [**A**] (0.336 to 3.25) x 10^{-2}
No correlation analysis made because the differences with the matching conditions (effect of plasma dilution, or of population group are obvious.				

[a] **Functions fitted**: to avoid premature selection of a given model, model-uncommitted polynomial functions were first fitted to the data for calculation basic properties and statistical analyses. We systematically tested polynomial functions of order 1 (linear), 2 (quadratic), or 3 (cubic) and selected the best-fitted function by visual inspection (geometric fit). If selecting between 2 functions proved difficult, we ran wo accepted tests to calculate their respective probability, namely the Extra sum-of-squares (using F test and calculating the p-value) and the Akaike's information criterion [corrected for small size samples] to calculate their respective probability. In most cases, both tests designated

the same function for all 4 sets of data in the same panel. The equations are

Y = B0 + B1∗X (1st order [linear]) and **Y = B0 + B1∗ X** + **B2∗X²** (2[d] order (quadratic)] were X is the total TMO concentration and B0, B1 'and B2) are the parameters defined in note [c].

[b] **groups**: #1: healthy individuals; #2: patients with UTI infection; #3: patients hospitalized in ICU with suspected/documented ventriculitis; #4: patients hospitalized in ICU with sepsis/septic shock. See Table 1 and Table S1 for all measured parameters and clinical characteristics of each group.

[c] **Parameters:**

n: number of data points used in the analysis (see note [e]);

R²: quality of the regression (see also note [f])

B0, **B1** (and **B2** if fitting a quadratic polynomial): function coefficients (best-fitted estimates). **B0** is the offset (**Y** value for **X = 0)**; for a 1st order (linear) polynomial function**, B1 is t**he slope. For a 2d order (quadratic) polynomial, **B1** and **B2** are the parameters determining the overall shape of the upward open parabolic function fitted to the data (B1 can tentatively be associated with an initial slope, and B2 to the progressive non-linear increase of Y).

[d] **Name** is how each set if data is referred to in the text when confusion could arise.

[e] **Number of data points:**

To ensure enough power for comparison analyses, all Y values, including those of replicates, were considered individually. For data from Figure 3, this number was constant across each type of study and is given in the corresponding heading.

[f] **Goodness of fit**: the R² parameter is reported as an indication of how well the function fits the data. Each regression analysis was further examined with the following diagnostic tools: (i) for all regressions, the value and homogeneity of the Sx.y. parameter and the normality of the residuals; (ii) for regressions using a 2[d] order polynomial (quadratic) function, we added a visual examination of plots of the residuals (X and Y, Q-Q) and plot of "Actuals *vs* Predicted" for all values). Regressions shown had no warning signal, showed Sx.y values compatible with

known errors and homogenous across groups or conditions, and did not show visual evidence of abnormal distributions for both Y and X residuals, QQ, and predicted *vs*. actual plots (most points close to the identity line with on evidence of systematic distancing reasonable distribution of individual points no evidence of systematic distancing).

[g] **ssD / 95% aCI:** symmetric Standard Deviation Regression: see notes [3] and [4] in Table 2).

[h] **plasma**: pooled plasma samples from donors of group #1; the data are organized in 4 sets with the same ranking as that of the clinical groups because the dilutions were calculated to obtain concentrations of proteins and albumin similar to those observed in individuals from each of these groups.

[i] dilution: made with 0.9 % NaCl

[j] the data are organized in 4 sets with the same ranking as that of the clinical groups because the concentrations of pharmaceutical albumin were calculated to obtain values similar to those of albumin observed in individuals from each of these groups.

Statistical analyses:

1. analysis by row (horizontal: comparing numeric values of the same parameter between the 4 sets of data in a given conditions; only the pertinent comparisons have been tested. Ordinary one- way ANOVA with Tuckey multiple comparisons test (comparing all groups; assuming equal standard deviations): values with different bold upper case letters are significantly different from each other ($p < 0.05$); similar results were obtained using Brown-Forsythe ANOVA or Welsch's ANOVA with and Dunnett's multiple comparison post-test (not assuming equal standard deviations). Letters have been ordered alphabetically (from A to D) starting with the column showing the lowest value, and moving to B, C, and D (if applicable) along the increase of the numeric value of the parameter analysed.
2. analysis by column (vertical: comparing the effect of different experimental approaches or different matrices). The differences of experimental conditions made point to point comparisons little informative. We therefore tested for correlation between the regression parameters of the functions (linear or quadratic) fitted to the data in the first condition with those of the functions fitted to the data of the other condition (analyses were made by parameter (including only those pertinent) and have been restricted to pertinent comparisons and when

the functions fitted to the data were of the same order [1st or 2^{d}];fitting functions of a different order was considered as indicating a significant difference between the two conditions for that reason]). For each comparison made, the text shown below the second condition included in the analysis gives a short description of what has been included in the correlation analysis and gives the values of the correlation coefficient (**r**) and its p value. We report only those comparisons for which the p-value was < 0.05 from parametric (Pearson's) statistics or non-parametric (Spearman's) statistics, depending on the normality distribution of the data.

Figures

Figure S1: Graphical representation of the administration of temocillin to subjects of each group (see Table 1 and Table S1) and from whom samples were collected to measure the total and unbound temocillin concentrations use to generate the data shown in the lower panels of Figure 1.

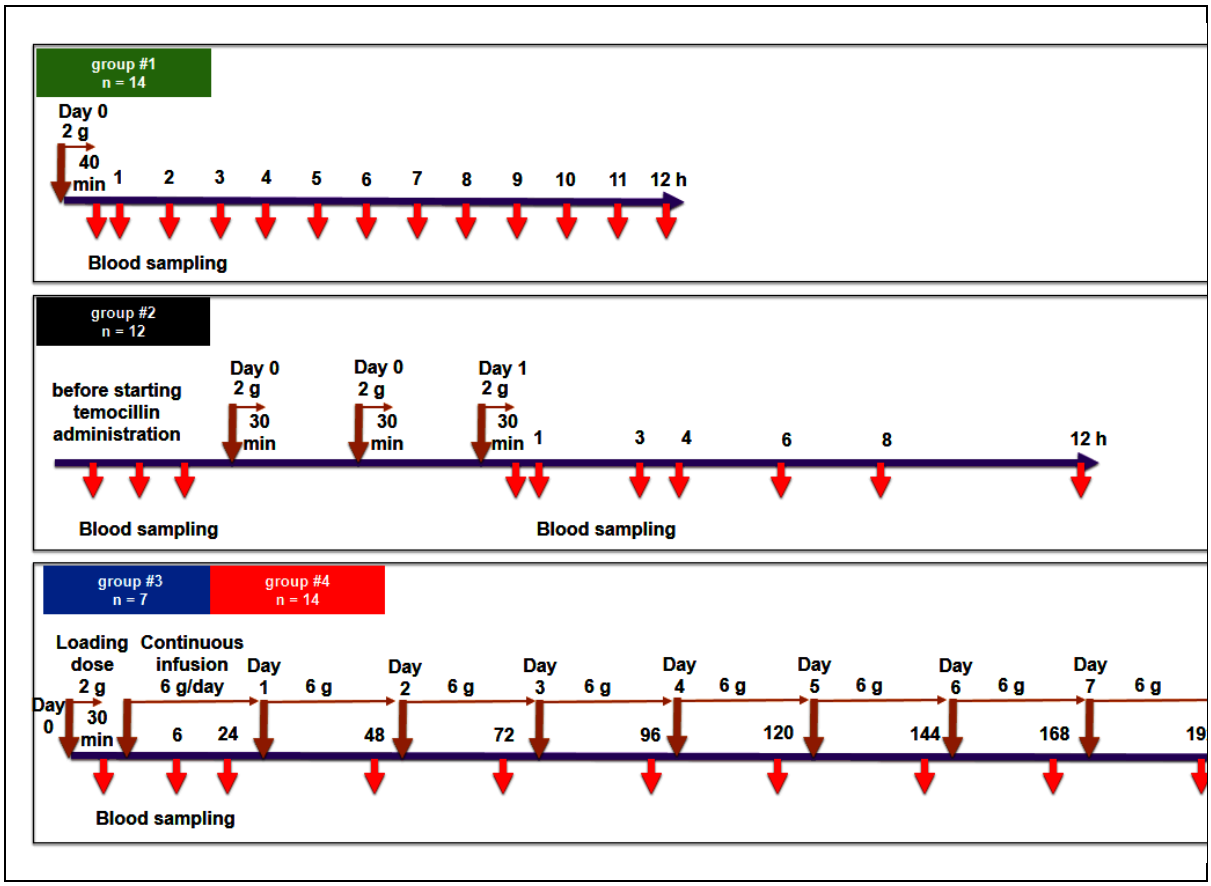


Figure S2-A. control study for temocillin binding to device. Samples with known concentration of temocillin in NaCl 0.9% were either assayed without contact with the ultrafiltration device) or after full contact with the device (addition, centrifugation and collection). The graph shows the concentrations observed (ordinate) as a function of the concentration in the samples as defined by the protocol of the study (theoretical concentrations). Data were analysed using simple linear regression analysis and the corresponding slopes and R^2 parameter are shown on the graph (deviation from linearity was not statistically significant [$p > 0.05$]).

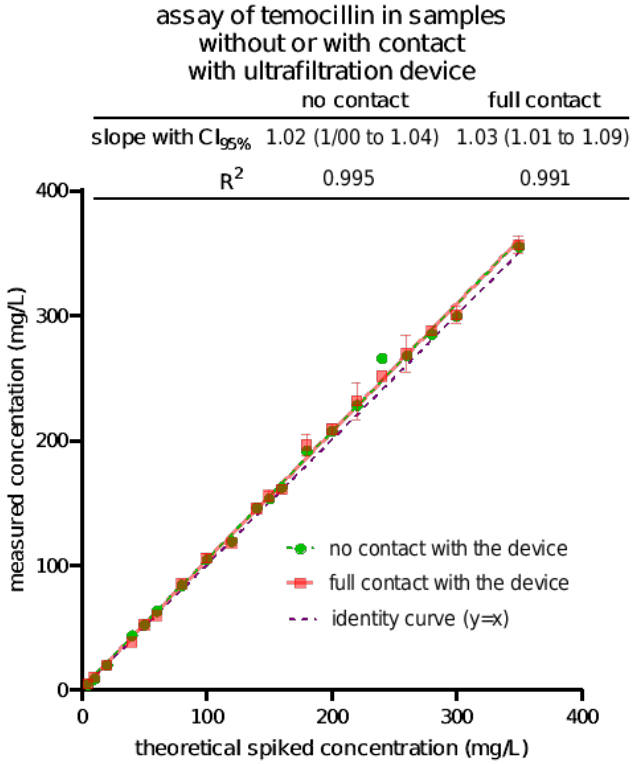
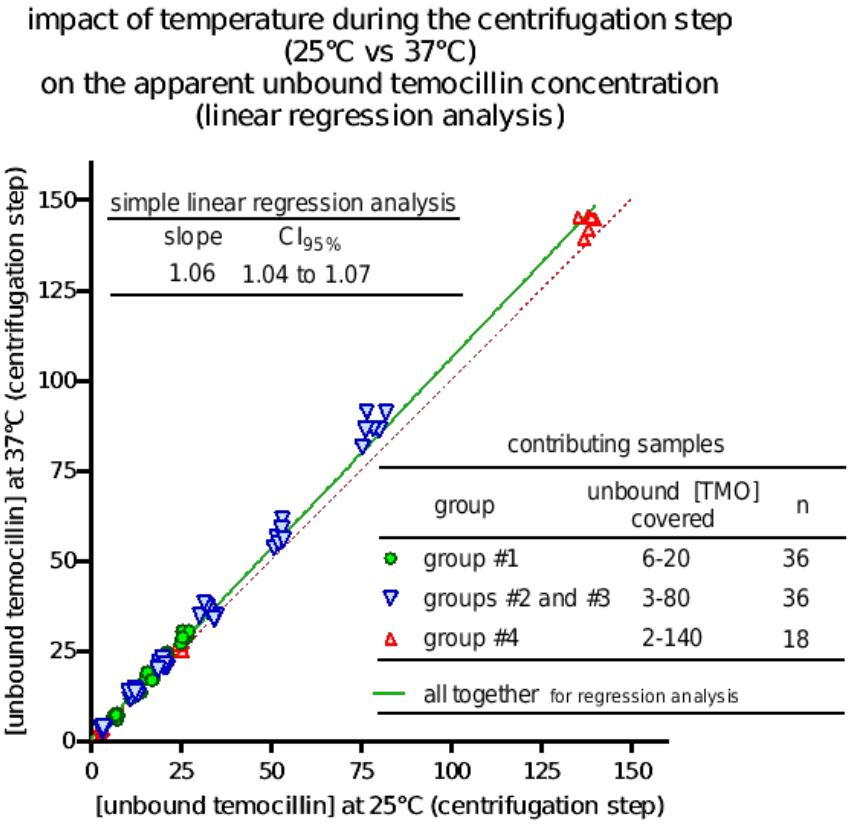
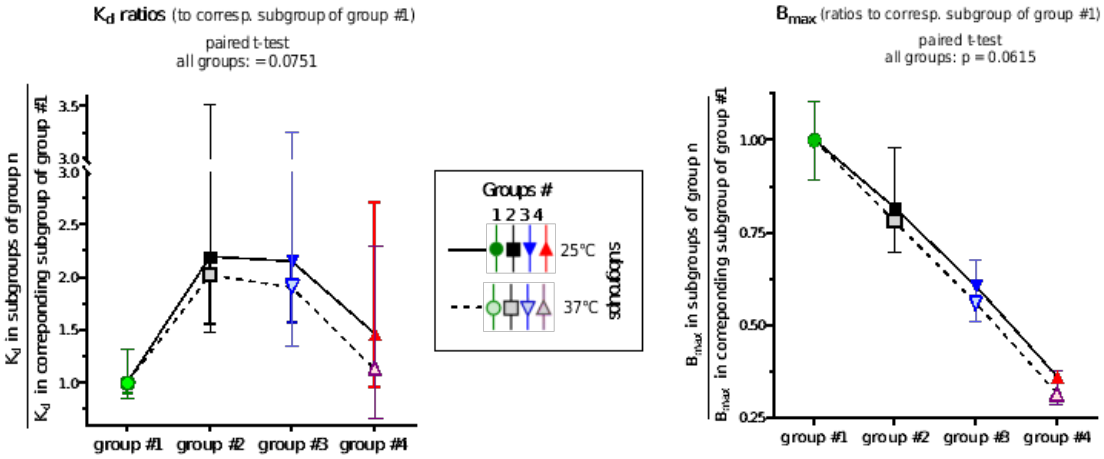


Figure S2-B. Correlation and regression analysis of temocillin unbound concentrations in samples from temocillin-naïve individuals belonging to groups contributing to this study (see Tables 2 and S1 for definition and characteristics of each group) and spiked with temocillin at a final total concentration known to generate unbound concentrations within the span of interest, taking into consideration the group of origin of each sample, and stratified according to the temperature at which the centrifugation step was performed.



The impact of temperature during the centrifugation step on the unbound concentration of temocillin recovered is minimal (slope factor: 1.06).

Figure S2-C. Calculated influence the centrifugation step temperature on the main values of the K_d (left panel) and B_{max} parameters. The values of K_d and B_{max} reported in Table S2 were used, but the ordinate is shown as the ratio of the value of the K_d or B_{max} parameter in subgroups of groups #2, #3 or #4 to the corresponding subgroup in group #1. Statistical analysis: t-test pairing samples from the same group to examine the global impact of temperature on the parameter examined (group 1 shows by definition a mean value of 1 for both subgroups).

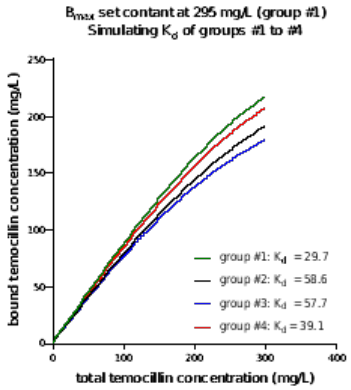


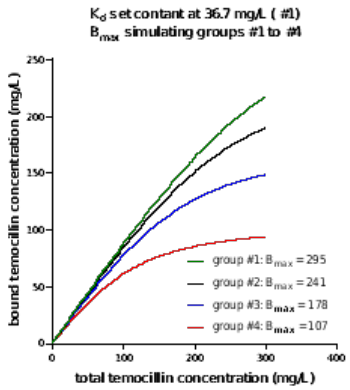
This figure highlights that the temperature applied during the centrifugation step of the samples in order to recover the unbound temocillin has no significant impact on the way K_d and B_{max} are changing among the 4 studied groups.

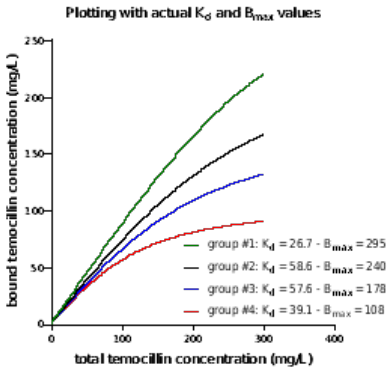
Figure S3. Simulations of the bound TMO concentration as calculated with the SBCALD equation and using the binding parameters K_d and B_{max} determined by curve fitting analysis. In these simulations, one parameter is kept constant while the other is set the values found by the curve fitting approach when examining different samples in order to visually document which parameter change causes most of the effect seen experimentally. In the examples shown here, we used (i) samples from donors coming from the populations shown in Table 1 and spiked with TMO (A1); (ii) samples from subjects coming from the 4 populations described above and who have been exposed to TMO (in-vivo; A2); (iii) plasma samples from a donor of group #1 undiluted or increasingly diluted to mimic the decrease of albumin concentration seen in donors of groups #1 to #4 (B). The left column shows the effect of a change in K_d (B_{max} remaining at its value in samples from group #1). The middle column shows the effect of a change in B_{max} with K_d remaining constant at its value in samples from group #1 or with undiluted plasma. The right column shows the effect of varying both K_d and B_{max}, as in the actual data reported in Figure 1C and F or in panel C of Figure 3. This set of simulations shows that changes in TMO binding related to the populations and infection are primarily related to changes in B_{max}. In contrast, changes related to plasma dilution are brought about by combined effects of a change in K_d and in B_{max}. In these studies, K_d and B_{max} are in drug-related units (mg/L).

A. Groups (#1 to #4)

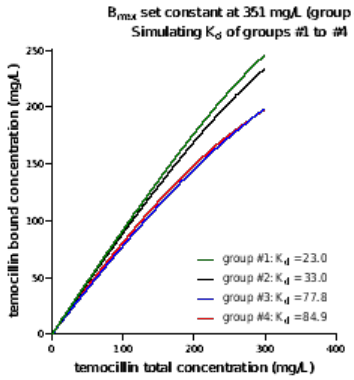
1. In-vitro

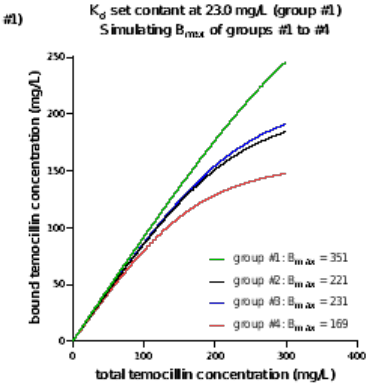


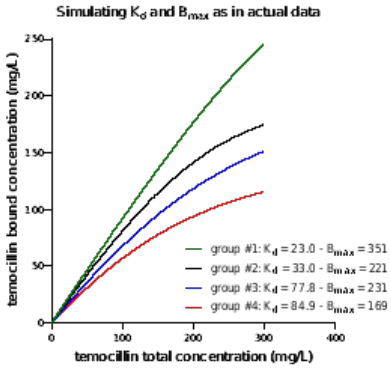




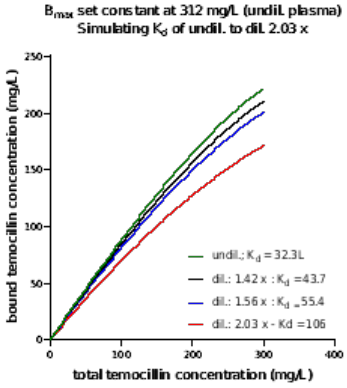
2. Ex-vivo

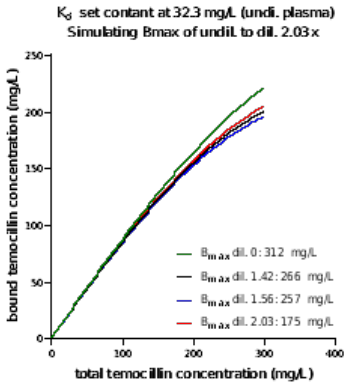






B. Plasma dilution (from undiluted to 2 x diluted)





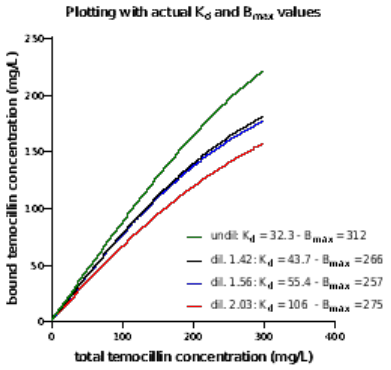


Figure S4: correlation (and linear regression) between unbound temocillin concentrations observed in samples from donors spiked with knows amounts of temocillin (whole population, abscissa) vs unbound temocillin concentrations in samples from healthy donors after increasing dilution with 0.9% NaCl (dilutions were made to mimic as much as possible the distribution proteins in the whole population of donors. The horizontal and vertical lines indicate the SD of each data point with respect to both axes and used for calculation of correlation parameters (Pearson's r = 0.977 (0.917 to 0.990); p< 0.0001). Regression to a 1st order polynomial equation (straight line) was made by non-linear regression and used only the SD of the data points along the ordinate (vertical lines) since this method assumes that variations affect only the ordinate value of each data point while its abscissa value is supposed to be known with certainty.
Equation of the regression: B0: 4.69 (1.69 to 7.69); B1 (slope) : 0.7633 (0.645 to 0.772); R^2 = 0.951.

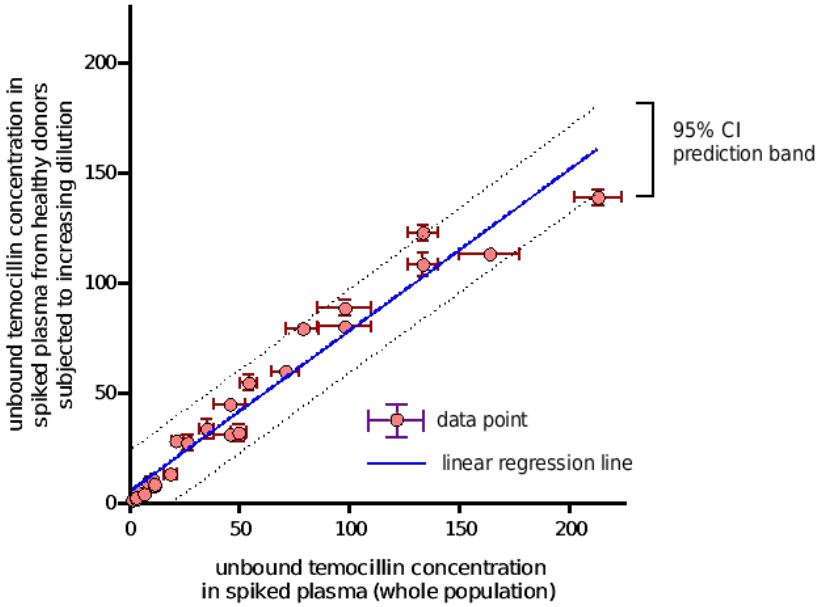


Figure S5: Left panel: Changes in the unbound fraction of temocillin in healthy volunteers' plasma samples spiked with a fixed concentration of competing drug. Temocillin was added at two selected total concentrations (50 and 150 mg/L) alone or in the presence of a fixed concentration of a competing drug (abscissa), used at a concentration most commonly observed in the plasma of patients receiving each of these drugs for therapeutic purposes and for its main indication (diazepam, 0.25 mg/L; midazolam, 0.25 mg/L; propofol, 4 mg/L; pantoprazole, 5 mg/L; tacrolimus, 0.01 mg/L; fluconazole, 20 mg/L). The ordinate shows the unbound fraction of temocillin, as determined experimentally. Statistical analysis: one-way ANOVA with multiple Dunnet's tests comparisons comparing the temocillin unbound fraction in samples spiked with temocillin plus each of these drugs to the unbound fraction of temocillin alone (control; p-value shown on top of each bar [*** if < 0.001]).

Right panel: Impact of competing drugs on the bound fraction of temocillin considering the whole concentration range of total temocillin concentrations investigated (20-300 mg/L). The ordinate is the bound temocillin fraction for each value total temocillin concentration investigated. The abscissa shows these concentrations of total temocillin expressed as the percentage of the maximal concentration tested value (300 mg/L). The curves were calculated using the SCBALD function fitted to the data as shown in Figure 4. The area under each curve gives the percentage of total temocillin contributed by the bound drug over the whole range of total concentrations investigated, taking into consideration its decreasing proportion when the total concentration increases. The figure emphasizes the decrease in the area under the temocillin curve when adding fluconazole, which dropped from 7610 to 6920, corresponding to a decrease of temocillin bound from 78.7 to 66.5 % of the total temocillin entered into the system (data for total temocillin concentrations < 20 mg/L (0 to 6.67 in the figure) have not been taken into account as the low signals recorded made them unreliable).

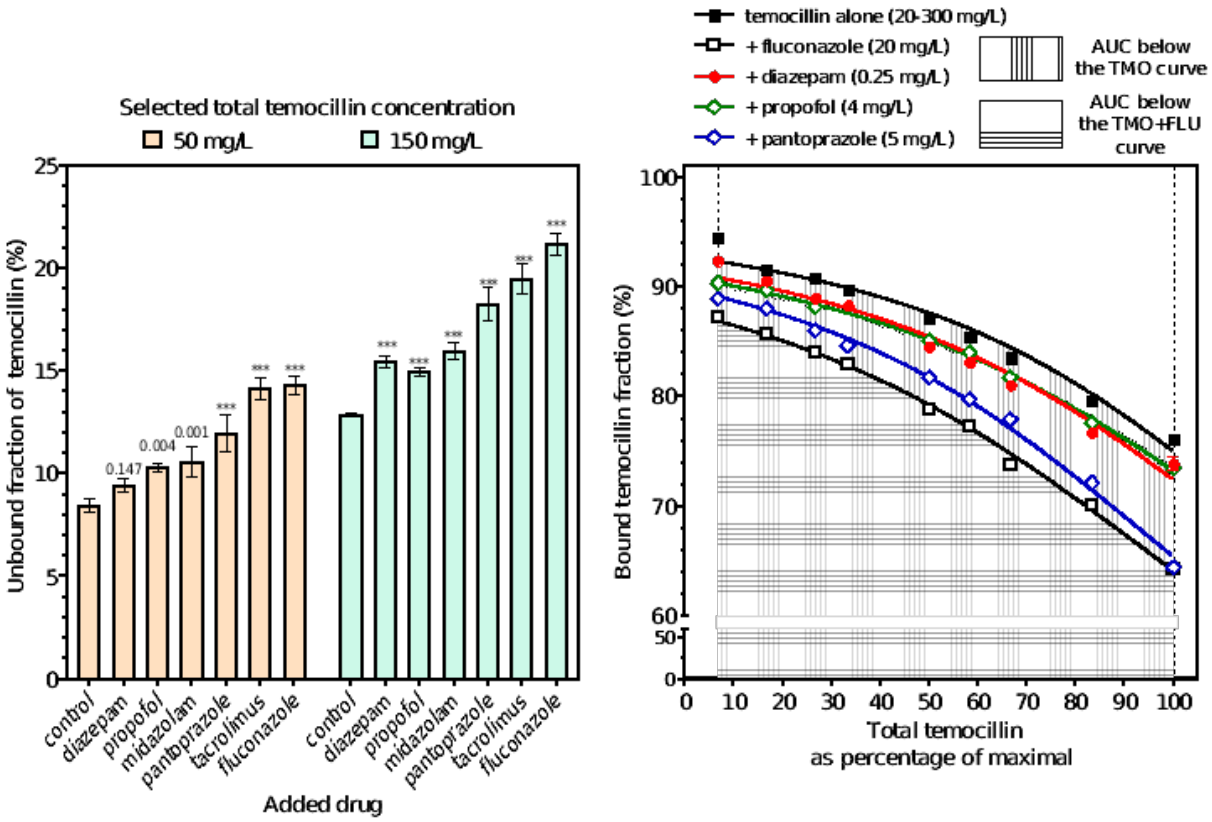

Selected total temocillin concentration
50 mg/L
150 mg/L
Unbound fraction of temocillin (%)
0.147
0.004
0.001
***
control
diazepam
propofol
midazolam
pantoprazole
tacrolimus
fluconazole
Added drug
temocillin alone (20-300 mg/L)
+ fluconazole (20 mg/L)
+ diazepam (0.25 mg/L)
+ propofol (4 mg/L)
+ pantoprazole (5 mg/L)
AUC below the TMO curve
AUC below the TMO+FLU curve
Bound temocillin fraction (%)
Total temocillin as percentage of maximal

Figure S6: correlation between measured and calculated temocillin unbound fractions at three total concentrations of total temocillin (50, 150, and 300 mg/L) when spiked alone or with competing drugs. The ordinate shows the measured unbound fractions. The abscissa shows the corresponding calculated unbound fractions using a modified SBCALD function (by multiplying K_d by $[1 + [i]/K_i]$ where [i] is the unbound concentration of the competing drug and K_i its dissociation constant from proteins (based on literature data; see details in Table S3 [Supplementary Material]) was used to calculate the bound concentration of temocillin for each of the concentrations without and with each of the competing drug, and the value used to calculate the corresponding unbound fraction. The Pearson correlation coefficient (Pearson's r factor) between observed and calculated unbound fractions was calculated first for each set of data corresponding to each of the 3 concentrations of total temocillin, and then globally for all data, and the values are shown in the figure. Likewise, orthogonal linear regressions (Demin Model II; selected because uncertainty affected both variables, which the classical linear regression analysis using least square minimization does not allow) were first made for each set of data and then globally for all data sets (see the corresponding regression lines in the Figure and the equation of the global regression line).

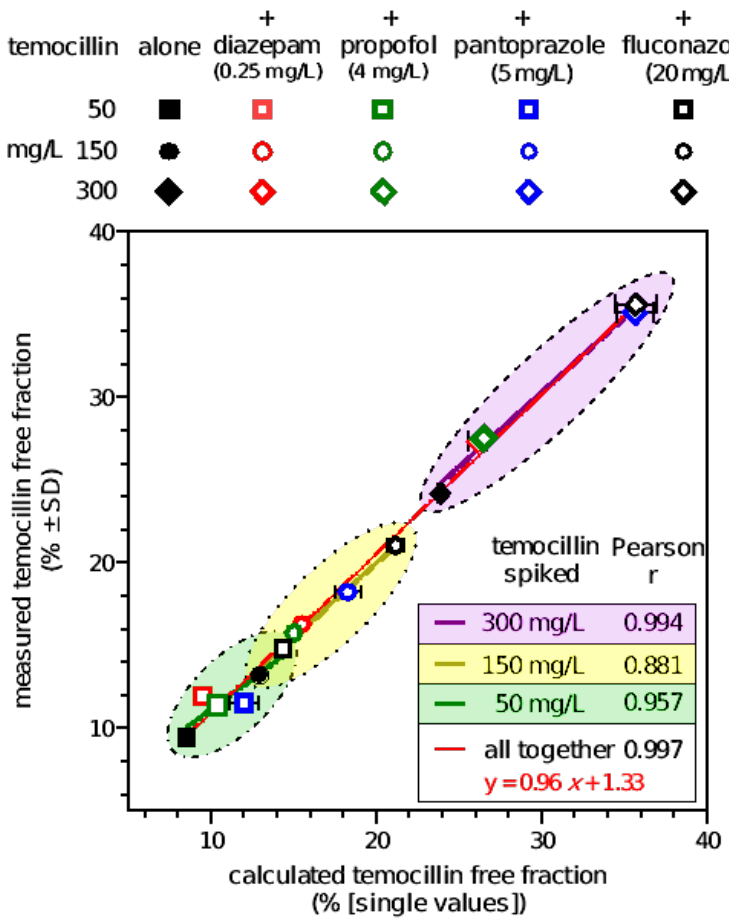
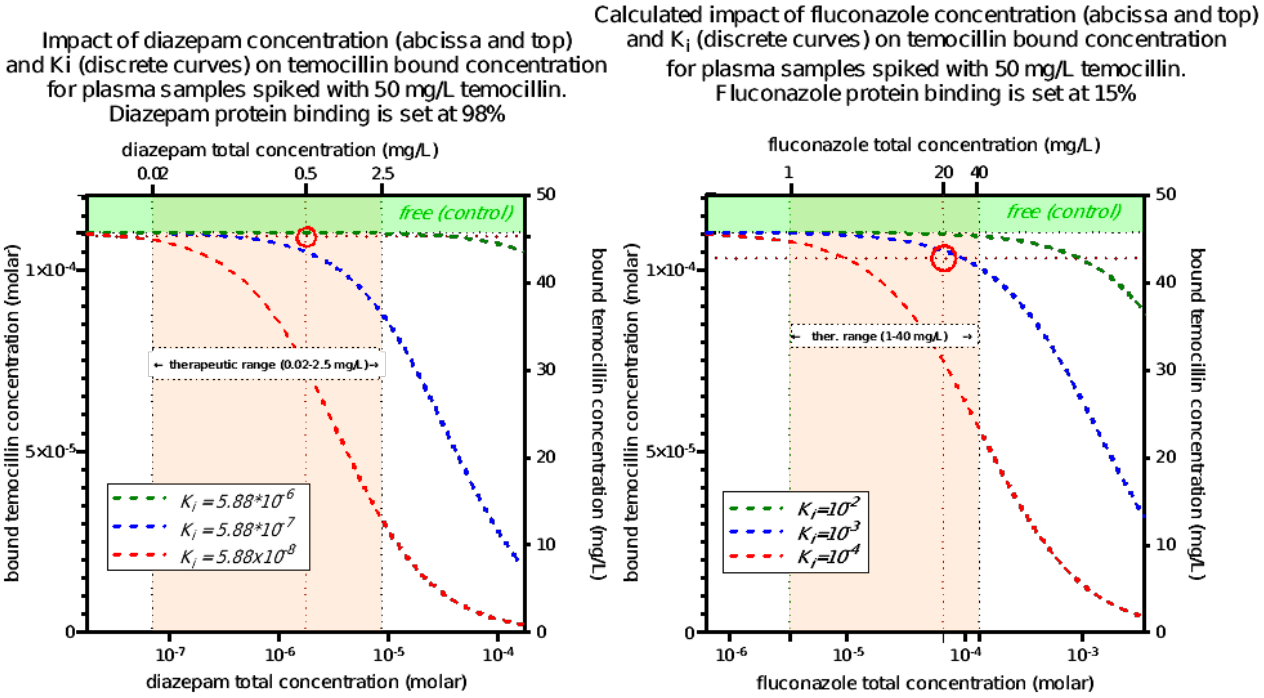


Figure S7 : simulations of the change in TMO bound concentrations for total concentrations of 50 mg/L in the presence of diazepam (left) or fluconazole (right) over a wide range of concentrations expressed either in molar concentrations (X axis below the graphs) or in mg/L (X axis above the graphs). Bound concentrations are also expressed in molar concentrations (left Y axis) or in mg/L (right Y axis). The orange zone corresponds to the clinically-relevant range of concentrations of diazepam or fluconazole and the dark red dotted line, to the concentration tested in competition experiments (see Figure S5-S7). The horizontal black line shows the bound concentration of TMO measured in the absence of competitor; the dark red dotted line, the concentration of TMO measured in the presence of competitor. Simulations were performed for three theoretical values of K_i for each drug, including the one that has been experimentally determined 5.88 10^{-7} and 10^{-4} to 10^{-3} ; for diazepam and fluconazole, respectively; see Table S3). The red circle corresponds to the conditions that were experimentally measured (see Figure 4C).



Appendix:

Modelling studies of temocillin protein binding

We developed a function allowing to determine by curve fitting analysis the best estimates of the key parameters (B_{max} and K_d) of the binding of a temocillin to plasma proteins (PP) using its bound and total concentrations as dependent and independent variables, respectively, under conditions of ligand depletion and partially correcting for difficulties related to the lack of experimental measure of the bound temocillin concentration.

Modelling of drug plasma protein binding (D-PPB) in its simplest form (fixed PP concentration, single site, no non-specific binding) often uses equation S1 (or equations that can be simplified to equation S2 [see ref[27,28] for review and ref[29] for a recent example]) to calculate the pertinent best-fitted parameters K_d and B_{max} :

$$C_{bound} = \frac{B_{max} * C_{unbound}}{K_d + C_{unbound}}$$

equation S1

Where C_{bound} is the concentration of the bound drug

B_{max} is the maximum concentration of drug that can bind to plasma proteins,

$C_{unbound}$ is the unbound (free) concentration of the drug

K_d is the apparent constant of dissociation of the drug-protein complex and correspond to the ratio of the rate constants of dissociation and association of the complex drug-plasma proteins

Equation S1 is actually a modified Hill-Langmuir equation (H-Le; isotherm or saturation binding curve) used in most pharmacological studies of ligand-receptor binding.[30-32] Its direct application to temocillin-PPB is, however, invalid for two reasons.

First, the H-Le is obtained from the equation describing the equilibrium between free ligand, free receptor, and their complex, by mean of a derivative that assumes the ligand concentration to be in large excess over that of the receptor.[30] This allows to use the total concentration of the ligand as a surrogate of its unbound concentration. Practically speaking, the free ligand concentration should never be lower than 90 % of its total concentration (rule of thumb).[33] This situation was not met here as we see that the unbound temocillin concentration is at most 73% of its total concentration, and on the average 20-30 % only, due to extensive binding to plasma proteins. Similar situations are observed in pharmacological studies when a ligand is in short supply and receptors abundant, and are referred to as ligand depletion situations,[30,34-37] calling for the use of a modified equation that takes this depletion into account.[30,38,39]

Second, the analytical techniques recommended in drug-plasma protein studies,[27,40] do not allow for an easy measure of the bound drug concentration although it is the dependent

variable in the H-Le. In most drug PPB studies, C_{bound} is calculated as the difference between C_{total} and $C_{unbound}$. This results in the same variable to appear in both arms of equation S1 when written explicitly:

$$(C_{total} - C_{unbound}) = \frac{B_{max} * C_{unbound}}{K_d + C_{unbound}}$$

equation S2

which violates a basic principle in curve fitting studies, leading to potential errors in the determination of best-fit values of the binding parameters.[38] This is shown graphically in the top panel of Figure A1. In the absence of experimental measure of C_{bound}, its value was calculated as the difference between the total and the unbound temocillin concentrations ($C_{total} - C_{unbound}$). Since $C_{unbound}$ is also used for the x-axis, its variations affect both coordinates. Replicates with different $C_{unbound}$ will not align them-selves on a vertical line, and will need to be considered as independent values for curve-fitting analysis. This makes impossible to assess the normality and intrinsic reproducibility of the raw data, also contradicting an important assumption commonly made in regression analysis, which is that the residuals from all data points are independent.

Inspired by pharmacological studies addressing the issue of ligand depletion,[32,38,39] we decided to develop an explicit form of the H-Le, to deliver a solution that could be more easily flowed and further developed.

We moved through the following steps:

1. posing: $C_{bound} = y$,
   $C_{total} = x$, and
   $C_{unbound} = x - y$,
2. writing a modified form of equation S2 : $y = \frac{B_{max} * (x-y)}{K_d + (x-y)}$
3. reorganizing this equation into a quadratic form

$$y * K_d + y * x + B_{max} * y - y^2 - B_{max} * x = 0$$

4. extracting the value of y to make the equation usable for curve fitting analysis by

- posing $a = K_d + x + B_{max}$, and
  $b = B_{max} * x,$
- allowing us to rewrite equation the quadratic equations as $-y^2 + a * y - b = 0$ which is an ordinary second-order polynomial equation, as shown by posing
  $Q = -1;\ R = a;$ and $S = -b,$
  which allows writing a differential equation: $\Delta = R^2 - 4 * Q * S$
  which is also $\Delta = a^2 - 4 * b$

- The latter equation has two solutions for $\Delta > 0$, namely

$$y_1 = \frac{-R-\sqrt{\Delta}}{2Q} \quad \text{and} \quad y_2 = \frac{-R+\sqrt{\Delta}}{2Q}$$

which can be rearranged into $y_1 = \frac{-a+\sqrt{\Delta}}{-2}$ and $y_2 = \frac{a+\sqrt{\Delta}}{2}$

5. Replacing a, b, Δ by their values (defined above), we obtain the two following equations:

$$y_1 = \frac{[-(K_d+x+B_{max})+\sqrt{((K_d+x+B_{max})^2-(4*x*B_{max}))}]}{-2}$$

and $$y_2 = \frac{(K_d+x+B_{max})+\sqrt{((K_d+x+B_{max})^2-(4*x*B_{max}))}}{2}$$

where $y1$ and $y2$ are the drug bound concentrations,
x is its total concentration, and
K_d and B_{max} are the binding parameters.

6. A plot of both functions shows that only the first one has y_1 in the expected range, with its value being 0 for $x = 0$.

7. The final equation used was, therefore:

$$y = \frac{[-(K_d+x+B_{max})+\sqrt{((K_d+x+B_{max})^2-(4*x*B_{max}))}\,]}{-2} \qquad \textbf{Equation S3}$$

(shown as equation 1 in the main text, and called **S**aturation **B**inding **C**urve **T**aking into **A**ccount **L**igand **D**epletion **(SCALD**).

A plot of actual data to use for fitting the SBCALD equation and determination of B_{max} and K_d parameters by curve fitting is shown in Figure A1 (middle panel [**B**]). Compared to H-Le, fitting of the SBCALD function was obtained with a better global quality and greater accuracy. The abscissa being the total rather than the free drug concentrations also allowed for an easier comparison of temocillin-PPB under conditions of different extent of binding such as those explored in the present paper.

Using the SBCALD equation, C_{bound} becomes a dependent variable, and $C_{total,}$ an independent variable. The ordinate (C_{bound}) still contains the variable used in the abscissa (since C_{bound} is C_{total}-$C_{unbound}$), but C_{total} is common to all replicates. Thus, all replicates will align vertically, making statistical analyses possible. Moreover, C_{total} is known with certainty for spiked samples, which was not the case of $C_{unbound}$). This makes each series of replicates independent from the others, a basic assumption in curve fitting studies. The issue of having the same variable on both sides of the equation is thus partially solved.

The binding parameters K_d and B_{max} are very similar whether using the H-Le or the SBCALD equations, which is expected as they are based on the determination of the binding in saturating conditions. A contrario, the calculated bound concentrations are markedly different below saturation, especially for samples from group #1 with high bound concentrations.

Limitations: The equations presented are limited to the analysis of one type of binding site and do not take into account non-specific binding, since this was sufficient for our study. Applications to the study of multiple binding sites and for the handling of non-specific binding would require additional mathematical work.

Other approaches

Over the years, two other approaches have been developed, with a first one proposed specifically for the study of drug PPB and a second to specifically address the issue of ligand depletion in pharmacological studies.

The first one, presented in 1976,[41] and applied very early on to the study of ceftriaxone[42,43] (another β-lactam with extensive and self-saturating PPB) as well as temocillin[44], favours the expression of the unbound drug concentration, which is then plotted against its total concentration using an equation bringing many similarities to ours. Figure A1 (lower panel [C]) shows a plot of the same data as those used for fitting the H-Le or the SBCALD equation. Although potentially providing similar information, we did not adopt this approach because it presents the data as if we were dealing with an accelerating process while we actually are facing a saturation one.

Addressing the issue of ligand depletion has been critical in pharmacological studies with the advent of transfected cells that express large amounts of the receptor under study. Combined with the limited supply of suitably labelled ligand (essential for the rapid measurement of its bound concentration) and the development of miniaturized assay techniques, this has increasingly created frequent situations where binding is no longer minimal and in which the free ligand concentration falls to values considerably lower than 90% if its total concentration.[35-37,45,46] Equations similar to ours, have been proposed and were a useful source of inspiration.[31,37-39,45] Software for specific pharmacological studies been made available.[33]

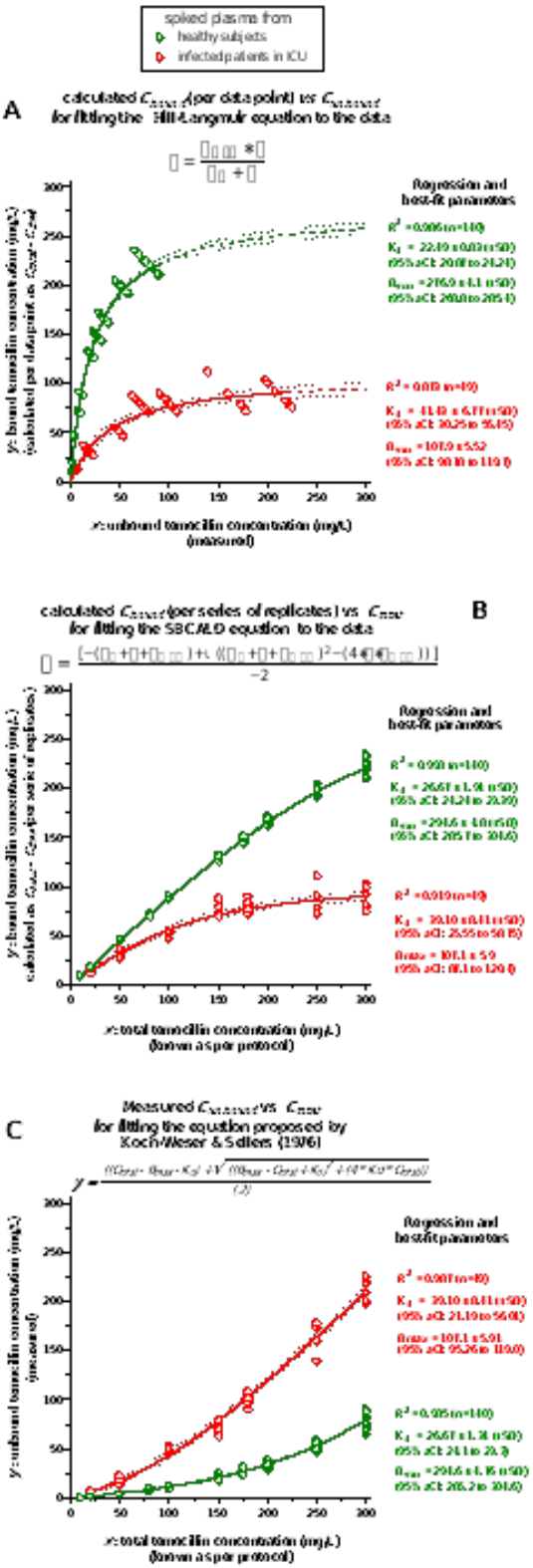
Lastly, we examined whether the implicit equation created by using $C_{total} - C_{unbound}$ as surrogate for C_{bound} in the H-L equation could not be used as such. Solving implicit equations require mathematical skills and may fail. Software has been developed in the context of pharmacological studies. The results, however, were disappointing as the available software yielded unreliable results unless pre-fed with the almost correct values of the K_d and B_{max} parameters, which defeated our attempt to rely on this approach in a first instance.

Figure A1: Curve fitting analysis of temocillin-PPB observed after spiking of plasma samples obtained from donors of group #1 (healthy) or from group #4 (hospitalized in ICU for sepsis/septic shock; see Table 1 in the main document for description of these groups). These were chosen here for illustration purposes because they represent extremes in temocillin-PPB maximal binding capacity; B_{max} parameter) among the populations examined. The regression parameters and best fit values of K_d and B_{max} parameters are shown on each graph. Curves are shown with 95% confidence interval (not clearly visible is very close or the actual curve).

Upper panel (**A**): Data are plotted using equation A1 (Hill-Langmuir): y axis: $C_{bound,}$ calculated as $C_{total} - C_{unbound}$ based on actual data of each sample. Since variations in C_{free} affects both coordinates, data points of a given replicate fail to align vertically and each value is therefore considered as an individual data point for the fitting of the Hill-Langmuir equation to the data. The dotted lines are extrapolations beyond the last experimental point up to an abscissa value of 300 mg/L to allow for direct comparison between data and equation fitting.

Middle panel (**B**): Data plotted using equation A3 (**SBCALD**). y axis: $C_{bound,}$ calculated as C_{total} - $C_{unbound}$. Since C_{total} has the same value for all samples within a given set of replicates, replicates will align vertically allowing to analyse each series of replicates and to consider each set of replicates as being independent and entered as such for fitting of the SBCALD equation to the data.

Lower panel (**C**) Data plotted using the equation proposed by Koch-Wezer & Sellers[41]: y axis: $C_{unbound}$ and x axis is C_{total}. Since the proportion of $C_{unbound}$ increases when C_{total} increases, the graph shows an increase for what is the result of a saturation.


spiked plasma from
healthy subjects
infected patients in ICU
A
calculated $C_{unbound}$ (per data point) vs $C_{unbound}$
for fitting the Hill-Langmuir equation to the data
$y = \frac{B_{max} * x}{K_d + x}$
Regression and best-fit parameters
R^2 = 0.986 (n=140)
K_d = 22.49 ± 0.83 (n=50)
(95% aCI: 20.88 to 24.24)
B_{max} = 276.9 ± 4.1 (n=50)
(95% aCI: 269.0 to 285.1)
R^2 = 0.88 (n=40)
K_d = 41.49 ± 6.17 (n=50)
(95% aCI: 30.25 to 56.45)
B_{max} = 101.9 ± 5.52
(95% aCI: 90.18 to 113.1)
y: bound temocillin concentration (mg/L)
(calculated per data point as $C_{total} - C_{free}$)
x: unbound temocillin concentration (mg/L)
(measured)
B
calculated $C_{unbound}$ (per series of replicates) vs C_{free}
for fitting the SBCALD equation to the data
$y = \frac{[-(B_{max} + K_d + x) + \sqrt{((B_{max} + K_d + x)^2 - (4 * B_{max} * x))}]}{-2}$
Regression and best-fit parameters
R^2 = 0.991 (n=140)
K_d = 26.67 ± 1.91 (n=50)
(95% aCI: 24.24 to 29.39)
B_{max} = 294.6 ± 4.8 (n=50)
(95% aCI: 285.1 to 304.6)
R^2 = 0.919 (n=40)
K_d = 39.10 ± 8.11 (n=50)
(95% aCI: 25.55 to 58.15)
B_{max} = 101.1 ± 5.9
(95% aCI: 88.1 to 120.0)
y: bound temocillin concentration (mg/L)
calculated as $C_{total} - C_{free}$ (average series of replicates)
x: total temocillin concentration (mg/L)
(known as per protocol)
C
Measured $C_{unbound}$ vs C_{free}
for fitting the equation proposed by
Koch-Weser & Sellers (1976)
$y = \frac{((C_{free} - B_{max} - K_d) + \sqrt{((B_{max} - C_{free} + K_d)^2 + (4 * K_d * C_{free}))})}{(2)}$
Regression and best-fit parameters
R^2 = 0.981 (n=40)
K_d = 39.10 ± 8.11 (n=50)
(95% aCI: 21.19 to 56.01)
B_{max} = 101.1 ± 5.91
(95% aCI: 95.26 to 113.0)
R^2 = 0.985 (n=140)
K_d = 26.67 ± 1.91 (n=50)
(95% aCI: 24.1 to 29.3)
B_{max} = 294.6 ± 4.85 (n=50)
(95% aCI: 285.2 to 304.6)
y: unbound temocillin concentration (mg/L)
(measured)
x: total temocillin concentration (mg/L)
(known as per protocol)

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