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Original article

A meta-analysis of protein binding of flucloxacillin in healthy volunteers and hospitalized patients

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ABSTRACT

Objectives: The aim of this study was to develop a mechanistic protein-binding model to predict the unbound flucloxacillin concentrations in different patient populations.
Methods: A mechanistic protein-binding model was fitted to the data using non-linear mixed-effects modelling. Data were obtained from four datasets, containing 710 paired total and unbound flucloxacillin concentrations from healthy volunteers, non-critically ill and critically ill patients. A fifth dataset with data from hospitalized patients was used for evaluation of our model. The predictive performance of the mechanistic model was evaluated and compared with the calculation of the unbound concentration with a fixed unbound fraction of 5%. Finally, we performed a fit-for-use evaluation, verifying whether the model-predicted unbound flucloxacillin concentrations would lead to clinically incorrect dose adjustments.
Results: The mechanistic protein-binding model predicted the unbound flucloxacillin concentrations more accurately than assuming an unbound fraction of 5%. The mean prediction error varied between −26.2% to 27.8% for the mechanistic model and between −30.8% to 83% for calculation with a fixed factor of 5%. The normalized root mean squared error varied between 36.8% and 69% respectively between 57.1% and 134%. Predicting the unbound concentration with the use of the mechanistic model resulted in 6.1% incorrect dose adjustments versus 19.4% if calculated with a fixed unbound fraction of 5%.
Conclusions: Estimating the unbound concentration with a mechanistic protein-binding model outperforms the calculation with the use of a fixed protein binding factor of 5%, but neither demonstrates acceptable performance. When performing dose individualization of flucloxacillin, this should be done based on measured unbound concentrations rather than on estimated unbound concentrations from the measured total concentrations. In the absence of an assay for unbound concentrations, the mechanistic binding model should be preferred over assuming a fixed unbound fraction of 5%. **Eveline Wallenburg, Clin Microbiol Infect 2022;28:446.e1–446.e7**

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Introduction

Flucloxacillin is a narrow-spectrum β-lactam antibiotic, frequently used for the treatment of Gram-positive bacterial infections [1,2]. There is an increased interest in dose optimization of β-lactam antibiotics. In critically ill patients, therapeutic drug monitoring (TDM) is recommended for this purpose [3–5]. The generally accepted target for efficacy of flucloxacillin is dependent on the time of the unbound drug concentration above the MIC of the targeted pathogen ($fT_{>MIC}$) [6]. For toxicity, a total concentration of 125 mg/L was reported in a retrospective study [7].

Generally, total drug concentrations are measured in clinical practice, where it is assumed that the unbound (pharmacologically active) fraction is similar for all patients over the whole concentration range. Measuring total concentrations is a technically more feasible and affordable option than measuring unbound concentrations, and is a generally accepted surrogate for measuring unbound concentrations.

Flucloxacillin is highly, and variably, bound to serum albumin in healthy individuals (95–96%) [1,8]. Protein binding is found to be lower in hospitalized and critically ill patients, with sometimes unbound fractions >20%, caused mainly by low albumin concentrations [9,10]. Furthermore, higher unbound flucloxacillin concentrations are associated with an increased unbound fraction [9,10], indicating that protein binding is saturable in the therapeutic concentration range.

Binding characteristics of flucloxacillin have been described previously in small study populations with mechanistic models [9–11]. So far, mechanistic characterization and evaluation of clinical flucloxacillin protein binding, with the aim of predicting unbound concentrations, have not been performed. Therefore, the objective of the current study was to develop a mechanistic protein-binding model to predict unbound flucloxacillin concentrations in different patient populations.

Methods

Data collection

For model development, we pooled four datasets from previously published studies by Gardiner *et al.* [12], Wilkes *et al.* [9], Jager *et al.* [10], and Wallenburg *et al.* [13]. The studied populations were healthy volunteers [12], non-critically ill [9] and intensive care unit (ICU) [10,13] patients. The fifth dataset, a study by Chin *et al.* [14], was not used for model building but for external validation, and contained data from hospitalized non-critically ill patients.

All datasets contained paired observations of total and unbound flucloxacillin concentrations, as well as information on serum albumin concentrations and the bioanalytical method used. For measurement of serum albumin concentrations, Chin *et al.* used a chromogenic assay with bromocresol green (BCG). The other studies used a chromogenic assay with bromocresol purple (BCP). Since the BCG assay leads to an overestimation of albumin, the albumin values of dataset 5 were corrected with a 5.5 g/L deduction [15,16]. The mechanistic binding model was developed independently of individual pharmacokinetic parameters or dose, as protein binding depends on concentrations and not on the dosing regimen. We converted all flucloxacillin and albumin concentrations in the dataset to molar equivalents.

Model development

The protein-binding meta-analysis was performed using nonlinear mixed-effects modelling with the software package NONMEM® (version 7.4.1) with the prediction ($PRED) subroutine. The first-order conditional estimation (FOCE) method with interaction between random effects and residual variability was used throughout model building. Inter-individual variability was assumed to be log-normally distributed. Residual error was modelled using a separate proportional error estimate for each dataset. Parameter precision was calculated using the sampling importance resampling procedure [17].

Since the aim of the mechanistic model was to predict the unbound concentration, this was assigned as the dependent variable. The unbound fraction (F_u) can be described by Equation (1) and the total concentration by Equation (2).

$$F_u = \frac{C_{unbound}}{C_{total}} \tag{1}$$

$$C_{total} = C_{unbound} + C_{bound} \tag{2}$$

Flucloxacillin is predominantly bound to albumin. Since flucloxacillin exhibits non-linear protein binding, F_u is dependent on the unbound concentration [18]. The total concentration can then be described by Equation (2).

$$C_{total} = C_{unbound} + \frac{B_{max} \times C_{unbound}}{K_d + C_{unbound}} \tag{3}$$

In Equation (3), B_{max} is the maximum binding capacity, and K_d is the equilibrium dissociation constant.

Finally, Equation (3) can be derived to predict the individual unbound fraction from the total concentration [19].

$$F_u = \frac{-K_d - B_{max} + C_{total} + \sqrt{(K_d + B_{max} - C_{total})^2 + (4 \times C_{total} \times K_d)}}{2 \times C_{total}} \tag{4}$$

The unbound concentration was calculated from the unbound fraction based on Equation (4) and the measured total concentration.

Structural model selection and covariate analysis were guided by physiological plausibility and the objective function value (OFV). A decrease of >3.84 points in the OFV, corresponding to a significance level of p 0.05 for nested models, was considered statistically significant in univariate testing. Serum albumin was tested as covariate on B_{max} using linear, exponential and power models. The used temperature during ultrafiltration for separation of the unbound flucloxacillin fraction was tested as binary covariate on K_d. K_d was estimated separately for the ultrafiltration temperatures of 25°C or 37°C.

Model evaluation

The predictive performance of the mechanistic model was evaluated and compared with the approach to multiply the measured total concentration by a fixed factor of 5% as unbound fraction. The population predictions were used for the evaluation, as the main question of our analysis was whether the unbound concentration could be predicted from the total concentration, without knowledge of the unbound concentration. The predictive performance for both the individual development datasets and the external validation dataset was determined.

The predictive performance was determined with the mean prediction error (MPE), the root mean squared error (RMSE) and the normalized RMSE (NRMSE). The NRMSE was calculated by dividing the RMSE by the mean observed unbound concentration. Confidence intervals for MPE were calculated as described previously [20]. Confidence intervals for RMSE and NRMSE were calculated as proposed by Faber [21].

With the final model, we evaluated whether predictions of unbound flucloxacillin cause incorrect dose adjustments and compared this to the assumption of an unbound fraction of 5%. Again, the fit-for-use validation was evaluated for both the external dataset and for the model-development datasets. For hospitalized non-critically ill patients a target of >0.5 mg/L was used for the unbound concentration. This was based on the generally accepted target of 100%$fT_{>MIC}$, assuming an MIC of 0.5 mg/L [22]. This MIC is the epidemiological cut-off value (ECOFF) from the MIC distribution of cloxacillin for methicillin-sensitive *Staphylococcus aureus* (MSSA) according to EUCAST: 0.5 mg/L [23]. The MIC distribution of flucloxacillin for MSSA is lacking, but is suggested to be similar to that of cloxacillin [24]. For ICU patients a target of >2.5 mg/L was used, based on a target of 100%$fT_{>5xMIC}$ and an MIC of 0.5 mg/L [5,6]. No threshold for toxicity was used, since there is no threshold for unbound flucloxacillin toxicity known.

Four different outcomes were possible:

1. The dose is correctly increased: both predicted and observed unbound concentrations are below the target.
2. The dose is correctly not increased: both predicted and observed unbound concentrations are above the target.
3. The dose is incorrectly increased: the predicted unbound concentration is below the target, whereas the observed concentration is above the target.
4. The dose is incorrectly not increased: the predicted unbound concentration is above the target, whereas the observed concentration is below the target.

Results

Datasets

The characteristics of the datasets used are shown in Table 1. These four datasets included a total of 710 paired observations of total and unbound flucloxacillin concentrations measured in 92 subjects. Dataset 2 contained no individual data on serum albumin concentrations. The dataset used for the external validation included 61 paired observations measured in 47 subjects.

Unbound flucloxacillin concentrations ranged from 0.0013 mg/L to 110 mg/L. Total concentrations ranged from 0.07 mg/L to 220.6 mg/L. A total of 3.5% of the measured concentrations were below the limit of quantification (BLQ). Considering the low frequency of BLQ data, we included these data in the analysis using the 'all data' approach as suggested by Keizer *et al.* [25]. The unbound fraction ranged from 2% to 72% and was typically higher in ICU patients than in non-ICU patients and healthy individuals. In line with what was previously found in the individual datasets, the unbound fraction increased with higher unbound concentrations (Spearman's correlation r 0.86, $p < 0.001$) and lower serum albumin levels (Spearman's correlation r −0.65, $p < 0.001$) (Fig. 1).

Model development

The mechanistic binding model was fitted to the observed data. Residual error was best described by a model with a proportional error for each of the development datasets.

Serum albumin was added as linear covariate for B_{max}:

$$B_{max} = \theta_{alb} \times serum\ albumin \quad (5)$$

In this equation, θ_{alb} is the estimated parameter describing the gradient in which B_{max} changes with serum albumin concentration. We found a θ_{alb} of 2.6 (95% confidence interval 1.4–3.8), indicating 2.6 molecules of flucloxacillin bind to 1 molecule of albumin. The introduction of serum albumin as covariate for B_{max} decreased the objective function with 86 points, corresponding with $p < 0.01$. The inter-individual variability in B_{max} decreased from 85.6% to 56.4%. Inter-occasion variability on B_{max} was tested, but could not be identified.

The temperature used during ultrafiltration was tested as binary covariate on K_d. K_d was 62.1 μmol/L at 25°C and 118 μmol/L at 37°C. Adding this covariate decreased the objective function value with 27 points, corresponding with $p < 0.01$.

Parameter estimates of the mechanistic protein-binding model are shown in Table 2. Eta and sigma shrinkages were <10%. Goodness-of-fit plots (Fig. 2) show that the model is appropriate for the data. The visual predictive check (VPC) for the final pharmacokinetic model shows that the distribution of observed concentrations is consistent with the distribution of the predicted concentrations.

Model evaluation

Results of the predictive performance of the final mechanistic binding model are presented in Fig. 3. The mechanistic binding model predicted the unbound flucloxacillin concentrations more accurately than assuming an unbound fraction of 5%. This means that the MPE of the mechanistic model was lower in all datasets. Furthermore, the predictions by the mechanistic model were more precise, as the (N)RMSE of the mechanistic model was lower in all datasets. The performance of the mechanistic model was less accurate in the ICU patients (dataset 1 and 4) than in the other populations, as is observed in the higher bias (MPE).

Table 1
Summary of used datasets

	Development datasets				Validation dataset
	Dataset 1 Wallenburg *et al.*	Dataset 2 Gardiner *et al.*	Dataset 3 Wilkes *et al.*	Dataset 4 Jager *et al.*	Dataset 5 Chin *et al.*
Subjects, *n*	33	12	31	16	47
Number of paired observations, *n*	232	264	136	78	61
Population	ICU patients	Healthy volunteers	Hospitalized patients	ICU patients	Hospitalized patients
Age, years	59 (30–83)	26 (21–38)	64 (21–91)	54 (20–76)	68 (18–87)
Male/female, %	73/27	58/42	77/23	50/50	70/30
Serum albumin, g/L	17 (8–31)	45 (NA)	28 (16–35)	21 (16–32)	30 (22–42)
Unbound flucloxacillin concentration, mg/L	9.69 (0.21–110.0)	0.26 (0.0013–2.78)	1.51 (0.05–35.0)	5.36 (0.076–29.5)	2.07 (0.038–30.3)
Bioanalytical method	LCMS	LCMS	HPLC	HPLC	LCMS
Separation method of unbound fraction	UF, 37 °C	UF, 37 °C	UF, 25 °C	UF, 37 °C	UF, 37 °C
Albumin method	BCP	BCG	BCP	BCP	BCG

Data are presented as median (range), unless noted otherwise.
BCP, bromocresol purple; BCG, bromocresol green.

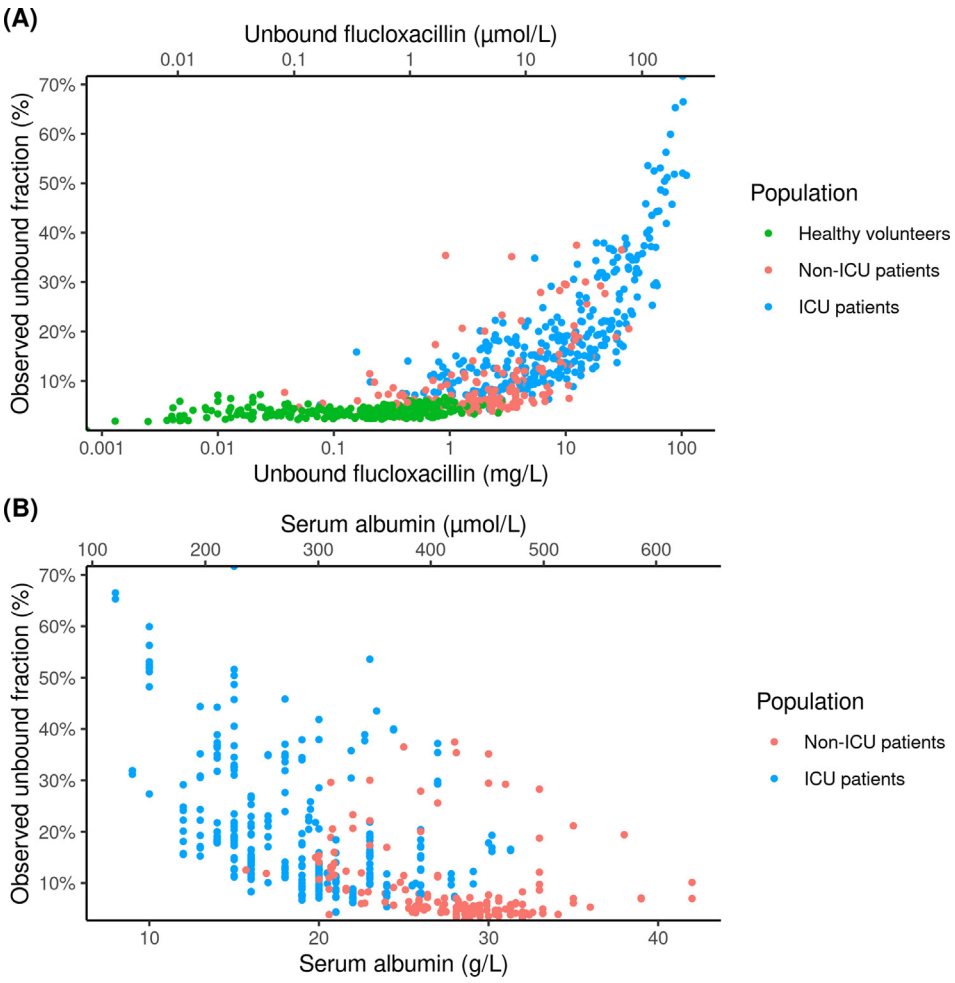

Fig. 1. Unbound flucloxacillin fraction versus unbound flucloxacillin concentrations (A) and versus serum albumin level (B).

Table 2
Parameter estimates of the base and final model

	Base model	Final model
B_{max} (µmol/L)	1000 (702–1298)	818 (426–1209)[a]
θ_{alb}	—	2.59 (1.35–3.83)
K_d (µmol/L)	94.6 (66.8–122)	62.1 (27.8–96.4)
25°C		118 (43.7–192)
37°C		
Interindividual variability (%):		
B_{max}	85.6 (71.3–105.3)	46.7 (39.0–55.2)
Residual proportional variability (%):		
Dataset 1	16.7 (15.0–18.7)	16.3 (14.9–18.7)
Dataset 2	20.6 (18.8–22.7)	20.7 (18.8–23.0)
Dataset 3	28.9 (25.2–33.2)	28.8 (24.9–33.6)
Dataset 4	16.5 (13.6–20.4)	17.5 (14.3–20.7)
Difference in objective function	Reference	−113

Data are presented as parameter estimate (95% confidence interval). [a] Estimated B_{max} for an individual with a median serum albumin level of 21 g/L, see Equation (5) in the text.

With the final model we evaluated whether predictions of the unbound concentrations cause incorrect dose adjustments and compared this to the assumption of a fixed unbound fraction of 5% (Fig. 4). More incorrect dose adjustments were made if a fixed ratio of 5% for the unbound fraction was used (19.4%) compared to the mechanistic model (6.1%). This concerns mostly incorrect dose increases. However, if the mechanistic model was used, the dose was more often maintained while a dose increase was justified (5.5% versus 2.2%).

Discussion

We performed a meta-analysis of the clinical protein binding of flucloxacillin. To our knowledge, we are the first to make use of a dataset of this magnitude, containing more than 700 paired observations from different patient populations. Our analysis confirmed the impact of flucloxacillin unbound concentrations, serum albumin concentrations and filtration temperature during the bioanalytical process on the unbound fraction. We showed that assuming a fixed unbound fraction, i.e. the current approach, is inferior compared to the mechanistic binding model to predict unbound concentrations in terms of accuracy and precision.

Our results show that, especially in critically ill patients, there is extensive inter-individual variability in the unbound fraction. Assuming an unbound fraction of 5% causes an underestimation of the unbound concentration in most critically ill patients, likely caused by hypoalbuminaemia in this population [26].

Despite the superior predictive performance of the mechanistic model, we dispute that even the mechanistic model is sufficient to use in practice to calculate unbound concentrations from total concentrations. We think that a bias (MPE) of more than 25% in critically ill patients and an imprecision (NRMSE) of more than 60% is unacceptable. Measuring the unbound concentration should be

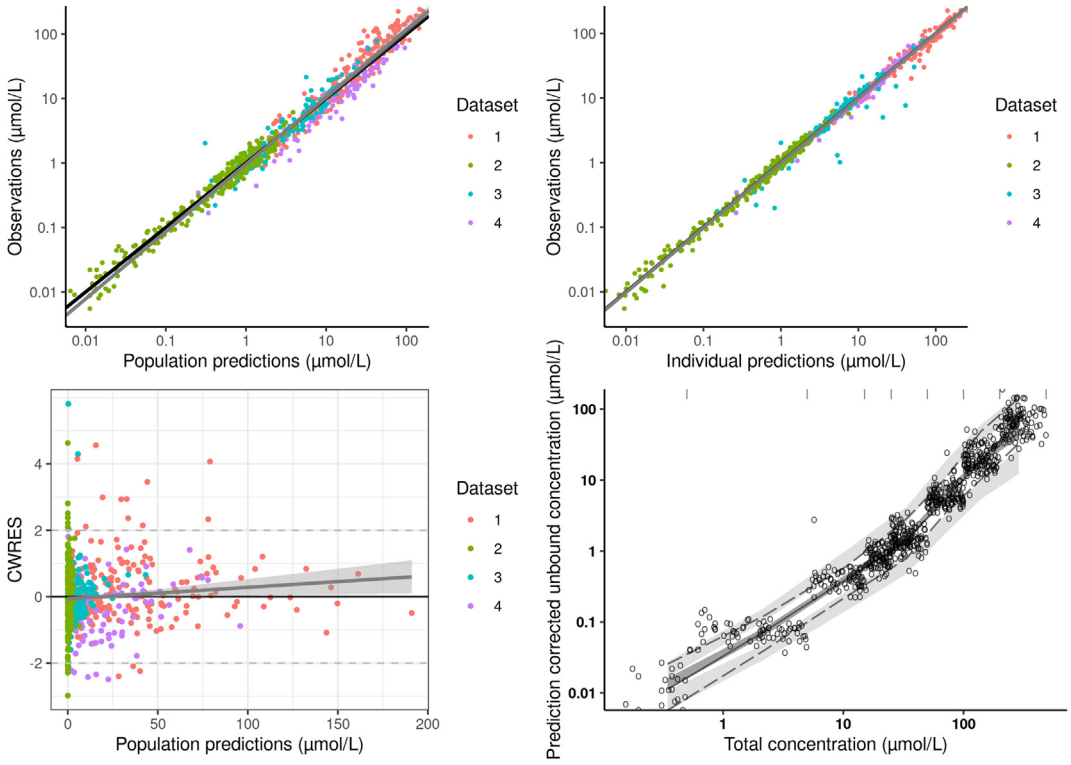

Fig. 2. Goodness-of-fit plots. Upper left panel: observed versus population predictions are evenly distributed around the line of unity. The different colours indicate the different datasets. Upper right panels: observed versus individual predictions are evenly distributed around the line of unity. The different colours indicate the different datasets. Lower left panel: conditional weighted residuals versus population predictions. The distribution of these residuals is homogeneous and the majority of the data lie within the interval (−2 to +2). Lower right panel: Prediction-corrected simulated (shaded) areas and observed (open circles) unbound concentrations versus total concentrations as independent variable in the model. The upper and lower lines connect the 5th and 95th percentiles of the observations. Light grey shaded areas are the 95% confidence intervals of the 5th and 95th percentiles. Dark grey shaded area indicates the confidence interval of the median. The distribution of observed concentrations is consistent with the distribution of the predicted concentrations, suggesting a good internal validity of the model to the data. For the higher total concentrations (>100 mg/L) the distribution of the observed concentrations is not completely consistent with the distribution of the predicted concentrations, suggesting less precision at very high concentrations, that are relatively rare.

preferred over the use of the mechanistic model. Moreover, the mean precision error (RMSE) varies between 0.13 mg/L and 12.5 mg/L and is in the same order of magnitude as is the therapeutic target (>0.5–2.5 mg/L), also underscoring the necessity of measuring unbound concentrations rather than calculating them.

The poor performance of the mechanistic model is caused by the relatively large unexplained variability in protein binding. There may be factors that we did not evaluate that could have explained part of this residual variability. For example, we did not evaluate the effect of co-medication that could cause drug displacement from albumin, since this information was not available. Flucloxacillin binds on Sudlow's binding site I and II of albumin [27]. Theoretically, drugs that bind these binding sites could displace flucloxacillin and alter binding properties. Also, we did not evaluate the effect of α-1-acid glycoprotein on B_{max}, since these data were not available. Although this covariate may partly explain variability in the unbound fraction [28], α-1-acid glycoprotein is not routinely monitored in clinical practice, which is a practical hurdle to predicting the unbound concentration of flucloxacillin in practice.

We showed that the binding affinity for flucloxacillin to albumin is higher at ambient temperature. For highly protein-bound drugs, ultrafiltration performed at ambient temperature might lead to an underestimation of the unbound fraction [29–31]. Ultrafiltration performed at physiological temperature (37°C) is likely to give a better representation of protein binding *in vivo* and is therefore recommended.

The differences in residual errors of the used datasets may be the result of differences in the analytical assay, as well as the sampling handling. The intra- and inter-day coefficients of variation were <10% for the analytical methods used in all studies. The residual errors are somewhat higher, but still in the same order of magnitude as the analytical error. Unfortunately, it remains unknown what exactly causes the differences in residual errors.

Both the mechanistic model and the use of a fixed unbound fraction to predict the unbound concentration might lead to incorrect dose adjustments in clinical practice. We found that the assumption of an unbound fraction of 5% will lead to more incorrect dose increases. Using the mechanistic model will result more often in maintenance of the current dose although a dose increase is necessary, and thus might cause unnecessary subtherapeutic exposure. One might argue that the risk of subtherapeutic exposure has greater clinical consequences than an incorrect dose increase. An incorrect increase in the dose might not be harmful, since flucloxacillin is thought to have a wide therapeutic window. On the other hand, underexposure might lead to treatment failure and contribute to the development of antimicrobial resistance [32–34].

The results of our analysis emphasize the need to measure the unbound flucloxacillin concentration if TDM is performed. For the purposes of TDM, it is relevant to assess whether inter-occasion variability in both pharmacokinetics and protein binding do not limit the utility of TDM [35]. In the current study, no inter-occasion variability in protein binding could be identified. Prospective studies are warranted to show that TDM based on the unbound fraction increases both the probability of target attainment and the rate of clinical cure. Also, the unbound threshold for toxicity should

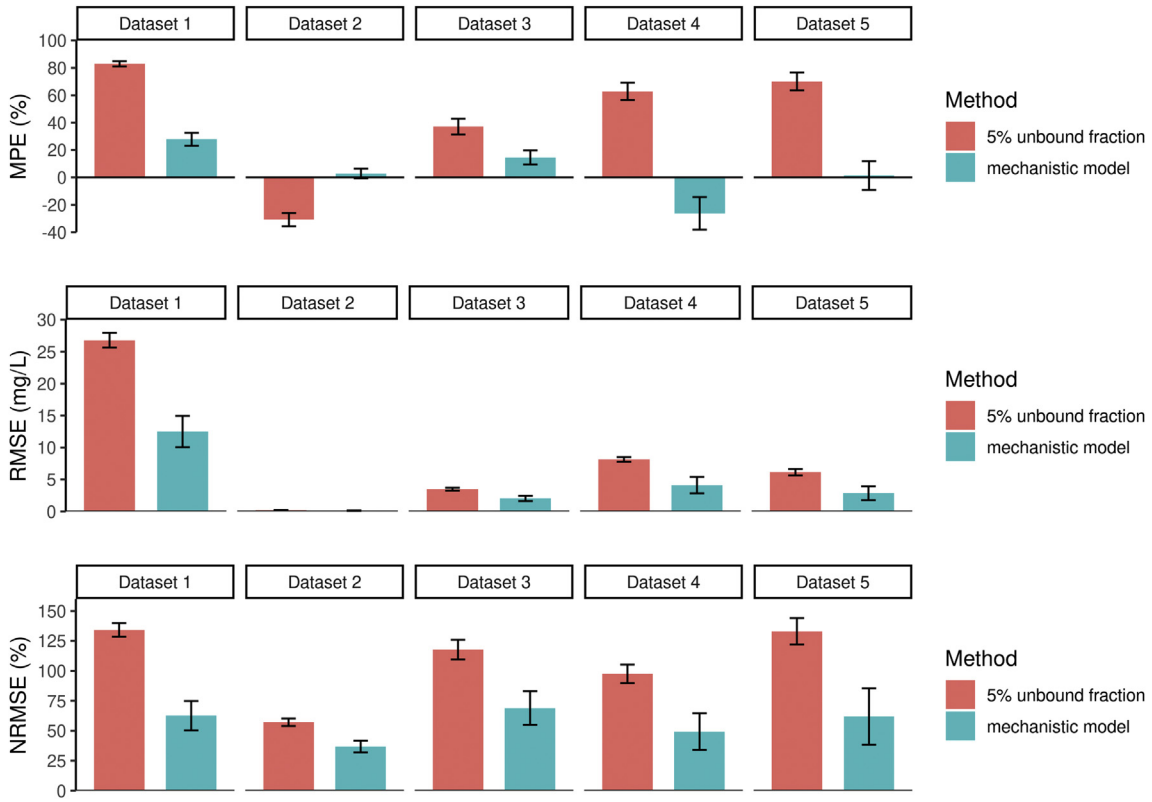

Fig. 3. Predictive performance of the mechanistic binding model and the method of using a fixed unbound ratio of 5%. Datasets 1–4 were used for the model development. Dataset 5 was used as external dataset for validation of the model. MPE, mean prediction error; RMSE, root mean squared error; NRMSE, normalized root mean squared error.

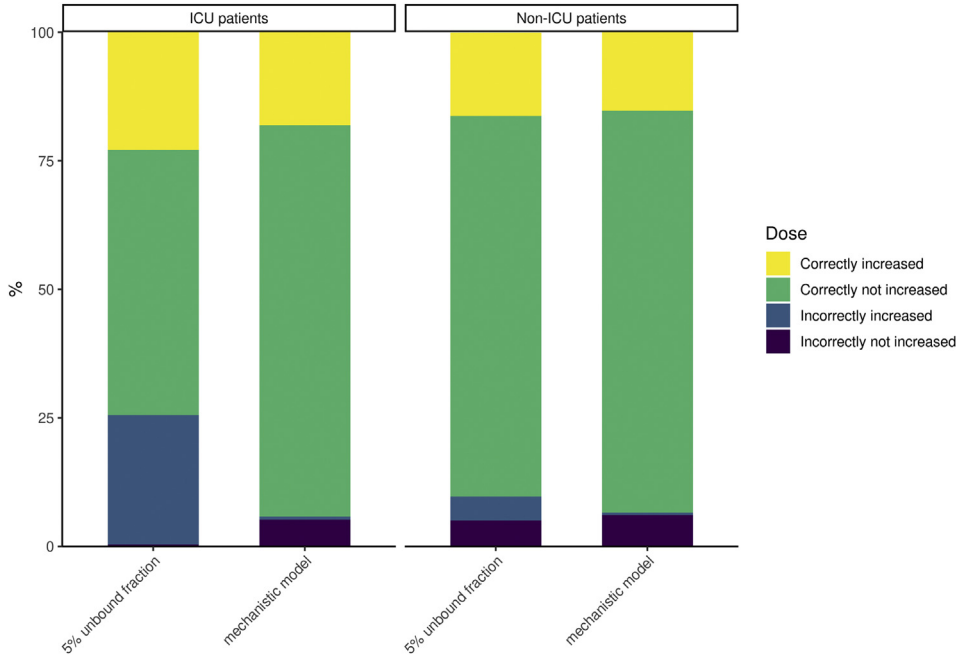

Fig. 4. Fit-for-use evaluation results.

be established to confirm the relationship between concentration and toxicity.

In conclusion, when performing dose individualization of flucloxacillin, this should be done based on measured unbound concentrations rather than on estimated unbound concentrations from measured total concentrations. In the absence of measured unbound concentrations, the best alternative is to predict the unbound concentration at 37°C using a mechanistic protein-binding

model rather than assuming a fixed 5% unbound fraction, but one should be aware of the limitations of this method.

Author contributions

EW, RtH and RB designed the study. JR, SW, JS and PC provided the data. EW and RtH analysed the data. All authors have read and approved the manuscript for publication.

Transparency declaration

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