Mind the time : 24-hour rhythms in drug exposure and effect
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CHAPTER

Identifying 24-hour variation in the pharmacokinetics of levofloxacin: a population pharmacokinetic approach

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SUMMARY

Aim: The objective of this study was to investigate whether the pharmacokinetics of orally administered levofloxacin show 24-hour variation. Levofloxacin was used as a model compound for solubility- and permeability-independent absorption and passive renal elimination.

Methods: In this single centre, cross-over, open label study, twelve healthy subjects received an oral dose of 1000mg levofloxacin at six different time-points equally divided over the 24-hour period. Population pharmacokinetic modelling was used to identify potential 24-hour variation in the pharmacokinetic parameters of this drug.

Results: The pharmacokinetics of levofloxacin could be described by a one-compartment model with first-order clearance and a transit compartment to describe drug absorption. The fit of the model was significantly improved when the absorption rate constant was described as a cosine function with a fixed period of 24 hours, a relative amplitude of 47% and a peak around 8:00 in the morning. Despite this variation in absorption rate constant, simulations of a once-daily dosing regimen show that T_{max}, C_{max} and the area under the curve at steady state are not affected by the time of drug administration.

Conclusion: The finding that the absorption rate constant shows considerable 24-hour variation may be relevant for drugs with similar physicochemical properties as levofloxacin that have a narrower therapeutic index. Levofloxacin, however, can be dosed without taking into account the time of day, at least in terms of its pharmacokinetics.

What is already known about this subject:

- The pharmacokinetics of drugs may show 24-hour variation, but this has rarely been systematically evaluated.
- Levofloxacin is an antibiotic whose oral absorption is limited by gastric emptying time and whose elimination occurs primarily via passive renal clearance, and can as such be used as a model compound to study 24-hour variation in these processes.

What this study adds:

- The absorption rate constant of levofloxacin shows considerable 24-hour variation, while other pharmacokinetic parameters seem constant throughout the day and night.
- This is relevant for drugs with similar physicochemical properties as levofloxacin that have a narrower therapeutic index, as the rhythm in absorption rate constant may be clinically relevant.

INTRODUCTION

Understanding the variables that influence the therapeutic effect of drugs is essential to optimize dosing strategies. One potential source of variation is introduced by the 24-hour rhythms in physiology, which are generated by an endogenous clock mechanism that is entrained to the 24-hour light-dark cycle and that allows us to anticipate to daily environmental changes (Mohawk et al., 2012). These rhythms are known to affect the pharmacokinetics, pharmacodynamics and toxicity of drugs (Dallmann et al., 2014).

Many physiological processes in the human body are subject to 24-hour fluctuations (Baraldo, 2008), such as gastric emptying time (Goo et al., 1987), hepatic enzyme activity (Takiguchi et al., 2007) and kidney function (Koopman et al., 1989). The complex interplay between these rhythms may lead to substantial variation in the pharmacokinetic parameters of a drug over the day and the night. With an increased understanding of the effect of these rhythms on the pharmacokinetics of a drug, the design of new and existing drug therapies can be improved by taking into account the optimal time of drug administration.

Scattered throughout the literature are a large number of studies that investigate the chronopharmacology of a wide variety of drugs, such as antibiotics (Beauchamp and Labrecque, 2007). These studies often employ a design that limits the interpretation and application of their results, thereby hampering implementation of the findings in the clinic. For example, as many chronopharmacological studies compare the pharmacokinetics following drug administration at two time points separated by twelve hours (Bleyzac et al., 2000; Choi et al., 1999; Fauvelle et al., 1994; Hishikawa et al., 2001; Rao et al., 1997), it is likely that the peak and trough are outside the studied intervals. Furthermore, most studies use an isolated approach in which the chronopharmacokinetics of one particular drug is investigated, without considering the relevance of their findings to other drugs with similar characteristics.

To overcome these limitations, a more systematic approach is required. For example, by investigating the chronopharmacokinetics of a model drug that represents a class of drugs that are absorbed, metabolized and/or eliminated in a similar manner, the findings can be extrapolated beyond the drug under investigation. Secondly, the use of multiple time points of drug administration is crucial in order to fully capture potential fluctuations over the 24-hour period. Thirdly, employing population pharmacokinetic modelling facilitates the identification of sources of variability related not only to the time of drug administration, but also to inter-individual and intra-individual differences, as utilized previously with midazolam (van Rongen et al., 2015).

The aim of this study was to investigate the chronopharmacokinetics of levofloxacin, an antibiotic characterized by solubility- and permeability independent absorption, minimal metabolism and passive renal elimination (Fish and Chow, 1997; Frick et al., 1998). Additionally, levofloxacin does not act primarily on the central nervous system, unlike many other drugs with similar physicochemical properties, so its influence on the central circadian clock in the hypothalamus is likely minimal. As such, levofloxacin was used as a model

compound to study the possible influence of 24-hour rhythms in physiological processes that determine the pharmacokinetics of many other drugs with similar properties. We developed a population pharmacokinetic model describing data from a clinical trial in which twelve healthy male subjects received an oral dose of 1000mg levofloxacin at six different time-points equally distributed over the 24-hour period. Simulations were performed to evaluate the effect of time of administration on several pharmacokinetic markers.

METHODS

Subjects

Healthy male subjects, aged between 18-50 years and with a body mass index (BMI) between 18-30 kg/m^2, were considered for inclusion. Eligibility was based upon results of medical history, physical examination, vital signs and laboratory profiles of blood and urine. Exclusion criteria included the use of concomitant medication two weeks prior to first drug administration until the end of the study, smoking and consumption of more than 21 units of alcohol per week or more than 8 units of caffeine per day. Subjects were also excluded if they were classified as extreme morning or evening types by the Horne-Ostberg morningness/eveningness questionnaire (Horne and Ostberg, 1976), if they were involved in transmeridian flights or shift work within a month prior to the start of the study until the end of the study or if they were otherwise unable to maintain a normal diurnal rhythm. All subjects provided written informed consent prior to the study. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and registered in the European Clinical Trials Database (EudraCT Number: 2013-001976-39).

Study design

This single centre, cross-over, open label study was carried out at the Centre of Human Drug Research in Leiden, the Netherlands. Subjects were randomly assigned to a treatment schedule consisting of six study visits separated by at least one week. A week prior to each study visit, subjects had to maintain a stable diurnal rhythm (waking times between 07:00 and 08:00, sleeping times between 23:00 and 00:00), which was verified by a sleep diary and a wrist-worn activity tracker (Daqtometer v2.4, Daqtix GmbH, Ötzen, Germany). Dietary restrictions included no caffeine or alcohol from 24 hours prior to drug administration and no dairy products or mineral fortified food supplements from 72 hours prior to drug administration.

Each study visit, subjects received an oral dose of 1000mg levofloxacin (Aurobindo Pharma B.V., Zwijndrecht, the Netherlands) with 200mL water at either 02:00, 06:00, 10:00, 14:00, 18:00 or 22:00 (Figure 1). Three hours after levofloxacin administration, subjects received an intravenous bolus of 5g inulin (250mg/mL; Inutest® from Fresenius Kabi, Zeist, the Netherlands). Subjects were fasted from t=-2h until t=6h. Subjects ate a maximum of four slices of bread at t=6h and a small snack at t=10h and drank at least 150mL water every 2 hours in order to keep fluid intake constant throughout the day and night. Between 23:30
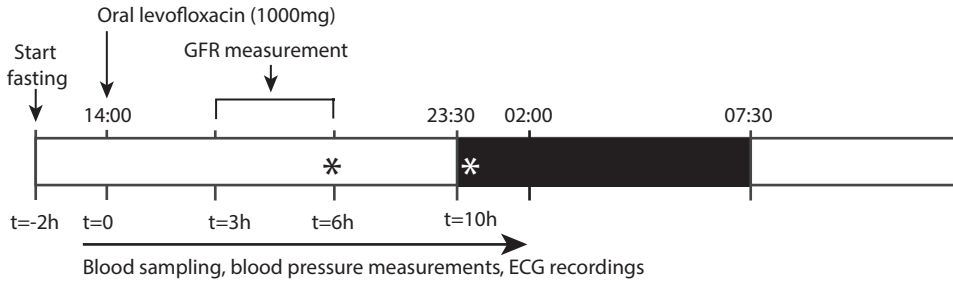
Figure 1 Schematic representation of a study occasion with drug administration at 14:00. Asterisks indicate the time-points the subjects were instructed to eat a standardized meal. In other occasions, the time of drug administration, measurements and meals occurred at a different time point (t=0 at 02:00, 06:00, 10:00, 14:00, 18:00 or 22:00), while the time the lights were dimmed and subjects wore an eye-mask were similar in all occasions (represented by the dark box from 23:30 until 07:30).

and 07:30, the lights were dimmed, subjects wore eye masks and sleep disturbance was kept to a minimum. Subjects remained in a semi-recumbent position from 30 minutes prior to dosing until the end of the study visit (except occasional toilet visits).

Blood samples (2mL) from an indwelling intravenous catheter and twelve-lead electrocardiograms (ECGs) were taken at predetermined time points (Table 1). Levofloxacin samples were collected in heparinised tubes, placed on ice and centrifuged at 2000g for 10 minutes at 4°C. Inulin and thyroid stimulating hormone (TSH) samples were collected in non-additive tubes. After coagulation for at least 45 minutes at room temperature, the samples were centrifuged at 2000g for 10 minutes at 4°C. All samples were stored at -80°C until further analysis. ECG recordings were stored using the MUSE Cardiology Information System. Because levofloxacin is known to prolong the QT interval, changes in QT interval were closely monitored during the study visits.

Levofloxacin

Acetonitrile protein-precipitation was used to isolate levofloxacin from plasma. Levofloxacin-d8 was added as internal standard. Chromatographic separation was performed on an XBridge C18 column using gradient elution. An API 4000 tandem mass spectrometer equipped with a Turbo Ion Spray probe operated in the multiple reaction monitoring (MRM) in positive mode was used for quantification. The lower limit of quantification (LLOQ) of this assay was 0.100 µg/mL. The inter-assay accuracy was between 101.1-111.0% and the inter-

Table 1 Sampling times

Analyte	Sampling times[a]
Levofloxacin	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12h
Inulin[b]	180, 185, 190, 195, 210, 240, 270 and 300 min
Thyroid stimulating hormone (TSH)	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11h
ECG recordings	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12h

a. t=0 defined as the time of levofloxacin administration

b. Inulin was administered at t=180min

assay variability was within 5.2%.

For non-compartmental analysis of the observed data, the maximal concentration (Cmax) and the time to Cmax (Tmax) were obtained directly from the individual data points. The area under the concentration-time curve from 0-12 hours after administration (AUC0-12) was calculated by the trapezoidal rule.

Glomerular filtration rate

Inulin concentrations in serum samples were determined by spectrophotometry. The determination was based on hydrolysis of inulin to fructose and formation of a purple-violet colour by fructose with ß-indolylacetic acid in concentrated hydrochloric acid. The LLOQ was 10.0 μg/mL. The inter-assay accuracy was between 103.3-110.2% and the inter-assay variability was within 8.0%. Systemic inulin clearance was calculated as the ratio of the dose to the baseline-corrected area under the curve from 0 to infinity using non-compartmental methods. To determine glomerular filtration rate (GFR), systemic inulin clearance was normalized by body surface area, calculated by the du Bois formula (Du Bois and Du Bois, 1916). Linear mixed effects modelling with the Nlme package in R (version 3.1.2, http://r-project.org) was used with GFR as the dependent variable, time of inulin administration as a fixed (categorical) effect and subject as a random effect. A likelihood ratio test of this model against the null model that included no fixed effect parameter was used to determine the effect of time of administration on GFR. P<0.05 was considered significant. Coefficients and 95% confidence limits of the full model were determined using the Effects package in R.

Thyroid stimulating hormone

Endogenous TSH concentrations in serum were measured by an electrochemiluminescence immunoassay (ECLIA, Cobas, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The LLOQ of this assay was 0.3mU/L. The inter-assay variability was lower than 0.6% and the intra-assay variability was lower than 1.6%. The relative TSH level per hour was calculated as follows:

$$\text{Relative TSH (\%)} = \text{TSH(t)}_i / \text{TSH}_i * 100\% \qquad \text{Equation 1}$$

where TSH(t)_i is the mean concentration of TSH of the ith subject at time t (sampling times were rounded to the nearest hour) and TSH_i is the mean of all TSH(t)_i values of the ith subject. The mean and 95% confidence intervals of the relative TSH levels per hour of all subjects combined was calculated and plotted against clock time.

Pharmacokinetic model development

A population PK model was developed to describe the concentration-time profiles of levofloxacin and to investigate the effect of time of administration on these profiles using nonlinear mixed effect modelling (NONMEM 7.3 (Beal et al., 2009)) in combination with Pirana (v2.8.2), PsN (version 3.7.6), Xpose (v4) and R (v3.1.2) to facilitate evaluation and graphical representation of the models (Keizer et al., 2013). Samples below LLOQ and

that were taken before Tmax were set to 0; samples that were below LLOQ and that were taken after the Tmax were omitted. The first-order method with conditional estimation and interaction (FOCEI) and the ADVAN6 subroutine was used throughout model development.

A stepwise approach was used to develop the population pharmacokinetic model. Different structural models (one- and two-compartment models) and the implementation of inter-individual variability (IIV) on the structural parameters were investigated. IIV was included according to equation 2:

$$P_i = \theta * e^{ni}$$

Equation 2

where P_i is the pharmacokinetic parameter for the ith individual, θ is the population pharmacokinetic parameter and η_i represents the IIV for the ith individual. Different models to describe residual error were tested (proportional, additive and combined). Various methods were used to characterize the absorption phase: zero-order absorption, sequential and parallel first- and zero-order absorption and first-order absorption with a lag-time and models with a fixed number of transit compartments or with an estimated number of transit compartments (Savic et al., 2007).

Next, several approaches to describe potential 24-hour variation in the pharmacokinetic parameters were assessed. Firstly, the 24-hour period was arbitrarily subdivided in equal sampling windows (e.g. for six equal sampling windows: window 1: 0:00-4:00, window 2: 4:00-8:00, etc.) and this was implemented as a covariate as follows:

$$\theta = \theta_{base} + \theta_{window}$$

Equation 3

where θ is the population pharmacokinetic parameter, θ_{base} represents the base value of the pharmacokinetic parameter (fixed to the value obtained in the model that did not contain this covariate) and θ_{window} represents the additive change in the pharmacokinetic parameter during that window. Secondly, IOV was included on the pharmacokinetic parameters as described previously (Karlsson and Sheiner, 1993), with each occasion representing a different dosing time. Thirdly, 24-hour variation in each of the pharmacokinetic parameters was evaluated by describing it as a cosine function with a fixed period of 24 hours as follows:

$$\theta(t) = \theta_{Mesor} + \theta_{Amp} * \cos(2\pi * (t - \theta_{\varphi}) / 24)$$

Equation 4

where $\theta(t)$ is the population pharmacokinetic parameter at time t (in hours after midnight), θ_{Mesor} represents the mesor (rhythm-adjusted mean), θ_{Amp} is the amplitude and θ_{φ} is the phase of the rhythm (corresponding to the time of peak in hours after midnight). If necessary, θ_{Mesor} was reparametrized to ensure the pharmacokinetic parameter remained positive during simulations (see below) as follows:

$$\theta_{Mesor} = e^{\theta trough} + \theta_{Amp}$$

Equation 5

where θ_{trough} is the value of the parameter at the trough of the cosine.

Covariate analysis was performed using a forward selection/backward elimination procedure. Continuous covariates (weight, height, lean body mass, body mass index, GFR and age) that showed a significant correlation (p<0.01, Pearson's correlation coefficient)
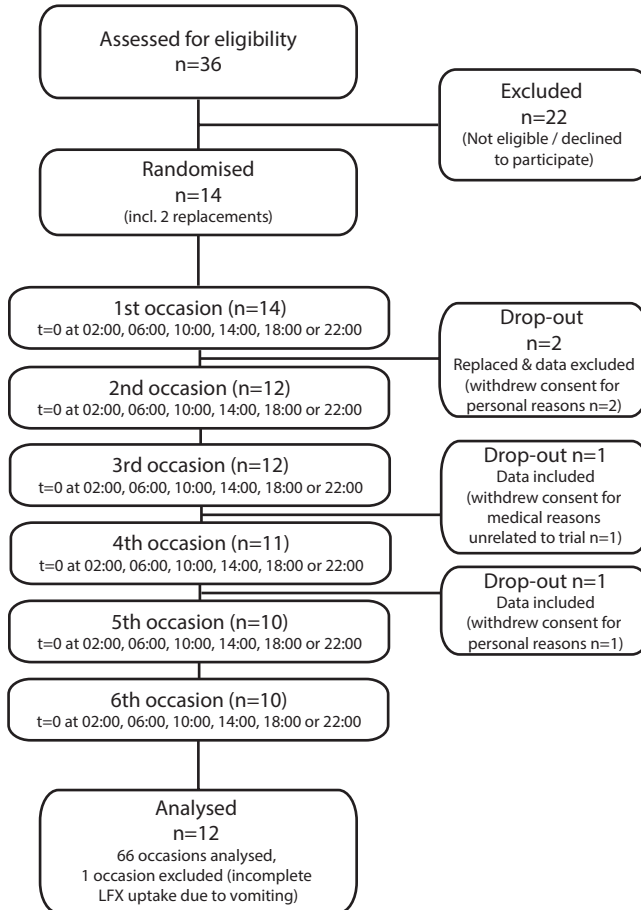
Figure 2 Clinical trial flow diagram. Twelve subjects were enrolled and randomly allocated to one of the treatment sequences in which drug administration occurred at a different time point in each occasion. Two of the twelve subjects withdrew consent after the first study visit. Their data was excluded and they were replaced. One subject withdrew consent after three occasions; another subject withdrew consent after four occasions. These subjects were not replaced and their data was included in further analysis. Data from one occasion had to be excluded due to incomplete levofloxacin (LFX) uptake after vomiting.

with a pharmacokinetic parameter were considered for inclusion in the model. Potential covariates were included as follows:

$$\theta_i = \theta_{pop} * (COV_i / COV_m)^{\theta COV} \qquad \text{Equation 6}$$

where θ_i is the covariate-adjusted pharmacokinetic parameter for the ith individual, θ_{pop} the population predicted pharmacokinetic parameter, COV_i the individual value of the covariate, COV_m the median value of the covariate in the population and θ_{cov} represents the covariate effect.

Model selection was based on objective function value (OFV), precision and plausibility of the parameter estimates (compared to previously published values of levofloxacin pharmacokinetics (Peloquin et al., 2008; Tanigawara et al., 1995; Zhang et al., 2009)), degree of shrinkage and graphical evaluation of the fit of the models (Mould and Upton, 2013). The

likelihood ratio test was used to compare the fit of nested models, under the assumption that the difference in -2 times log likelihood is chi-square distributed with degrees of freedom (df) determined by the number of additional parameters. Hence, a model in which the OFV decreased at least 6.63 points (p<0.01) upon inclusion of one additional parameter was considered to provide a significantly better fit of the data than the parent model. A visual predictive check (VPC) based on 1000 simulated individuals and stratified on the time of administration was performed to determine how well the observed data is captured by the final model.

Simulations

Simulations were performed using the package deSolve (v1.11) in R. To obtain the Cmax, Tmax and AUC_{0-12} of the observed data (see above) to the model predicted data, the individual predicted parameter estimates were used to simulate the concentration-time profiles of the 12 subjects (sampling every minute from t=0 until t=12h). Concentration profiles of a once-daily 1000mg dose administered at different dosing times for seven days in 500 subjects were simulated using the fixed and random parameter estimates of the final model and the uncertainty around the fixed parameter estimates and the C_{max}, T_{max} and the AUC during the dosing interval at steady state (referred to as $C_{max,ss}$, $T_{max,ss}$ and AUC_{ss}) were computed.

RESULTS

Subjects

A total of 66 occasions from 12 subjects were available for analysis (Figure 2). The demographics of the study population are summarized in Table 2. The treatment was generally well tolerated, although several adverse events (AEs) were reported, including headache (10% of the occasions), nausea (5.8%) and dizziness (5.8%). On several instances, the QT interval slightly increased after levofloxacin administration, but no QT-related AEs were reported.

Table 2 Overview of subject demographics

	N	Mean	Range
Age (years)	12	28.0	21.0-48.0
Body mass index (kg/m2)	12	24.0	19.4-29.2
Height (cm)	12	186	179-192
Weight (kg)	12	83.5	66.7-105
Lean body mass (kg)	12	61.1	55.2-69.3
Glomerular filtration rate (mL/min/1.73m²)	64[a]	114	86.2-174

a. Two GFR values were missing due to problems with the administration of inulin. These values were replaced with the median value of GFR values from other occasions of this subject for covariate analysis.
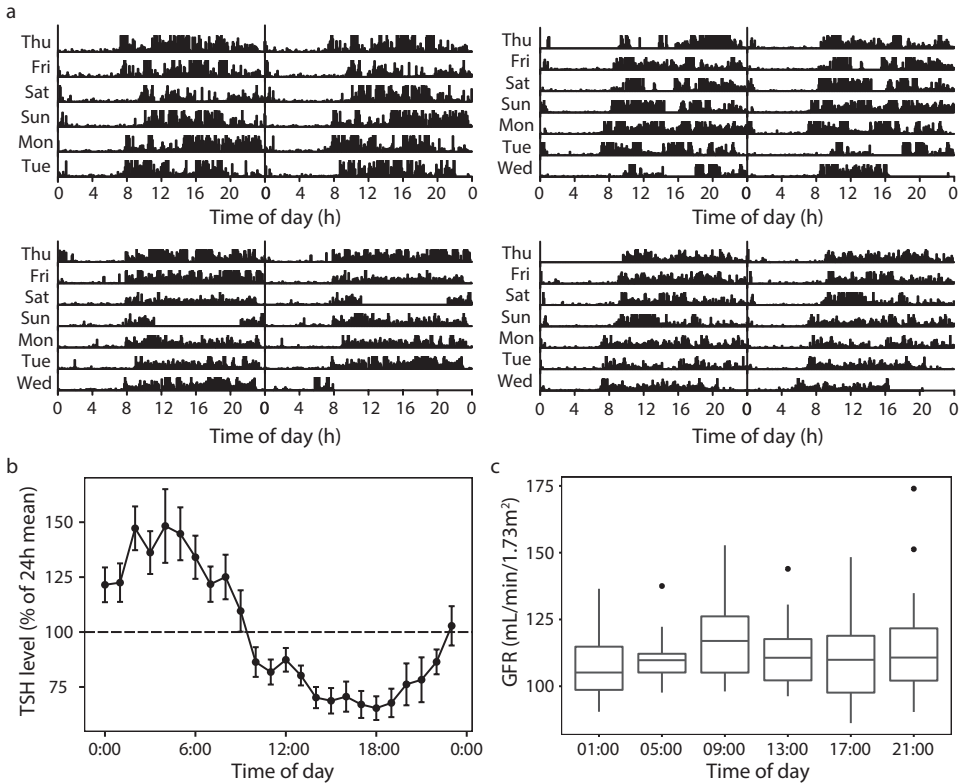
a

b

c

Figure 3 Rhythms in physiological parameters. **(A)** Four representative actograms from different subjects collected one week before a study visit. Days are double-plotted for clarity. **(B)** Mean relative change in thyroid stimulation hormone (TSH) levels over the course of the 24-hour period in all subjects combined (error bars: 95% confidence intervals). Time of sample was rounded to the nearest hour. **(C)** Boxplots showing the distribution of glomerular filtration rate (GFR) at six time-points during the 24-hour period. Upper and lower hinges encompass the inter-quartile range (IQR); upper and lower whiskers extend to the highest and lowest value within the 1.5*IQR; points represent data beyond the whiskers.

Physiological parameters

Actigraphy data was collected a week prior to each study visit. Seventy percent of the actograms could be generated successfully and indicate that the subjects maintained a constant behavioural rhythm as instructed (Figure 3A). Furthermore, thyroid stimulating hormone (TSH) levels, a rhythmic marker that was collected hourly during the study visits, exhibited clear 24-hour rhythmicity with peak serum levels occurring between 02:00 and 05:00 at night in the population (Figure 3B) as well as on an individual level (Supplemental Figure 1). GFR showed small time of day variation (Figure 3C). Linear mixed effects modelling indicated that time of day significantly affected GFR ($\chi^2(5)=11.8$, p=0.038). The highest GFR was observed when inulin was administered at 09:00 in the morning (estimate [95% CI]: 118 [108-129] mL/min/1.73m^2) and lowest at 01:00 at night (108 [97-119] mL/min/1.73m^2), amounting to a maximal difference of 9%.
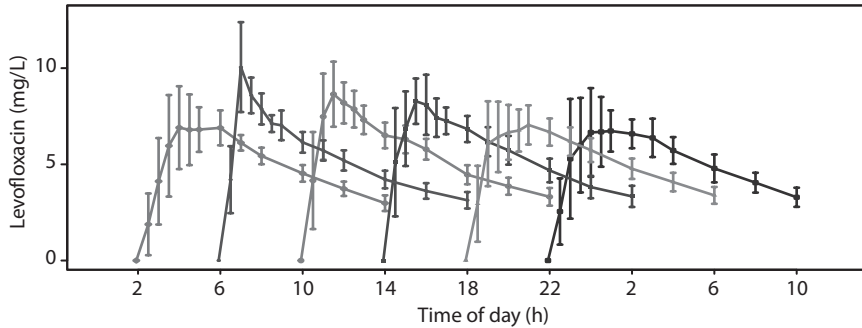
Figure 4 Concentration time profiles of levofloxacin in plasma per time points of administration. Raw data is presented as mean ± 95% CI.

Model development

Seven hundred ninety-two post-dose concentrations of levofloxacin were available for pharmacometric analysis. Nineteen samples (2.4%) were BLOQ. Mean concentration-time profiles are shown in Figure 4. When comparing different structural models, it was found that a one-compartment model with first-order absorption and elimination described the data well. Adding a second compartment did not improve the fit compared to the one-compartment model (ΔOFV -0.362). Inter-individual variability (IIV) could be identified on the absorption rate constant (Ka), apparent clearance (Cl/F) and central volume of distribution (V/F). A proportional error structure was used to describe the residual random variability. The absorption phase could be best described by adding a transit compartment with the transit rate constant (Ktr) equal to Ka. Covariance between IIV on Cl/F and V/F was included in this model.

Subsequently, it was investigated whether any of the pharmacokinetic parameters exhibited 24-hour variation. Firstly, we used six parameters (Eq. 3) to describe the effect of the time window during which a sample was taken on the pharmacokinetics of levofloxacin. This allowed us to explore the presence and shape of the 24-hour variation in the parameters, despite the relatively long half-life of the drug. Applying this approach to Cl/F, V/F or Ka resulted in a change in OFV of respectively -3.39 (p>0.01), -14.9 (p>0.01) and -52.4 (p<0.01, 5df). Hence, the fit of the model significantly improved when the effect of sampling window is included on Ka. Although the precision of these estimates of θ_{window} was low (RSE: 45-253%), a pattern was revealed that resembles a sinusoidal curve with higher Ka values during the early morning and afternoon and lower values during the evening and night (Figure 5A). A similar pattern was found when IOV was included on Ka (Figure 5B).

Because of the sinusoidal profile that was identified in Ka, it was attempted to describe this parameter as a cosine function (Eq. 4), with the mesor parametrized using Equation 5 and IIV included on θ_{trough}. Inclusion of the cosine on Ka reduced OFV by -84.8 points (p<0.01, 2df) and has similar goodness of fit (Supplemental Figure 2) compared to the parent model (without variation in Ka). Of note, the shape of the cosine on Ka resembles the pattern that was found when IOV or the effect of sampling window was included on Ka (Figure 5C).
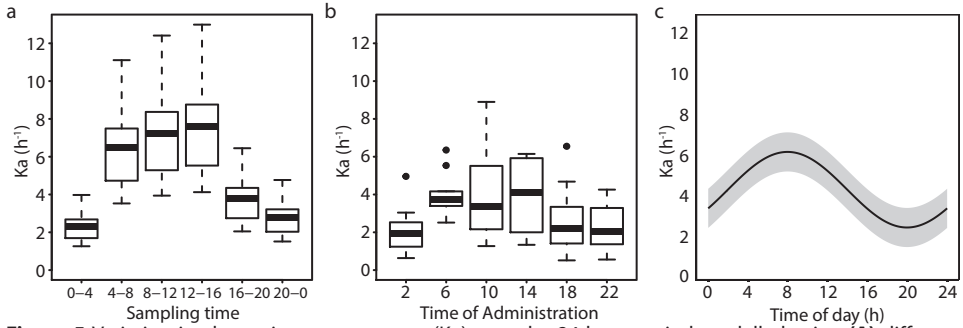
Figure 5 Variation in absorption rate constant (Ka) over the 24-hour period modelled using **(A)** different additive terms depending on the time winding during which the sampling was performed, **(B)** interoccasion variability on the different times of administration and **(C)** the estimated cosine function with a fixed period of 24 hours. Grey area: 95% confidence interval of the individual predicted curves (including interindividual variability on θ_{trough}).
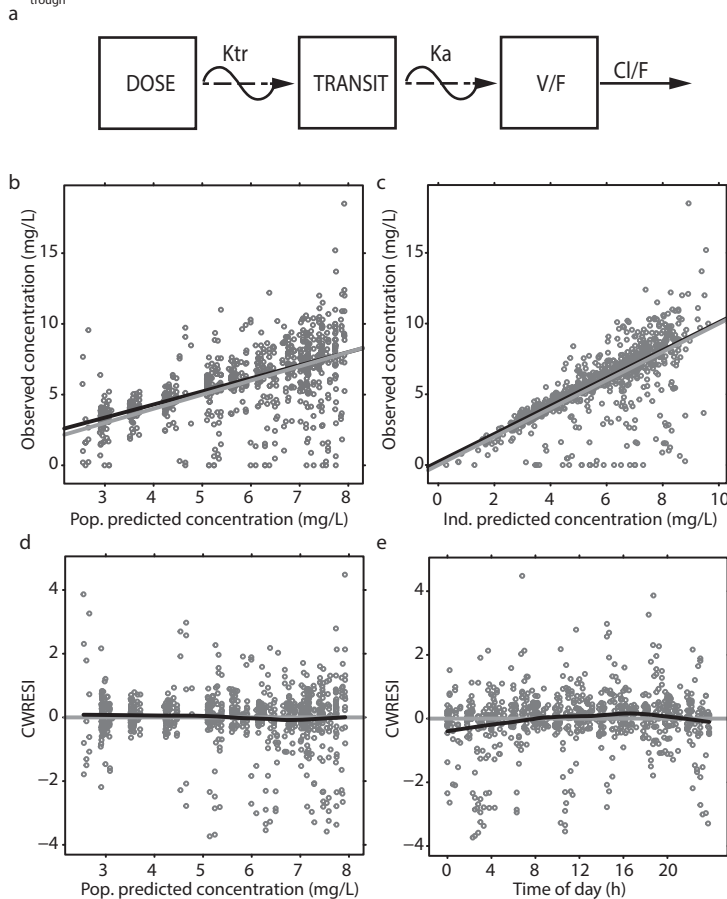

Figure 6 Structure and fit of the final model. **(A)** Final model structure: a one-compartment model with linear absorption and clearance (Cl/F), one transit compartment and a cosine function to describe the absorption rate constant (Ka). V/F: volume of distribution; Ktr: transit rate constant. **(B-C)** Observed versus population predicted concentrations (B) and individual predicted concentrations (C). Light green lines: line of unity, black line: linear regression line. **(D-E)** Conditional weighted residuals with interaction (CWRESI) versus population predicted concentrations (D) and time of day (E). Light green lines: horizontal line through y=0; black line: LOESS curve with span=0.6

Therefore, this one-compartment model with one transit compartment (with Ktr=Ka) in which Ka was described as a cosine function with a period of 24 hours (referred to as the "cosine Ka model") was used for further model development (Figure 6A). Covariate analysis showed that none of the covariates that were tested (LBM on Cl/F, V/F and the mesor of Ka, age on the mesor of Ka and GFR on V/F and Cl/F) significantly improved the fit of the model.

The parameter estimates of the cosine Ka model are shown in Table 3. The population and individual predicted concentrations of the cosine Ka model describe the observed concentrations accurately (Figure 6B and C) and the conditional weighted residuals (CWRESI) are symmetrically distributed around zero, without substantial concentration- or time-dependent bias (Figure 6D and E). η and ε shrinkage were below 3%. A visual
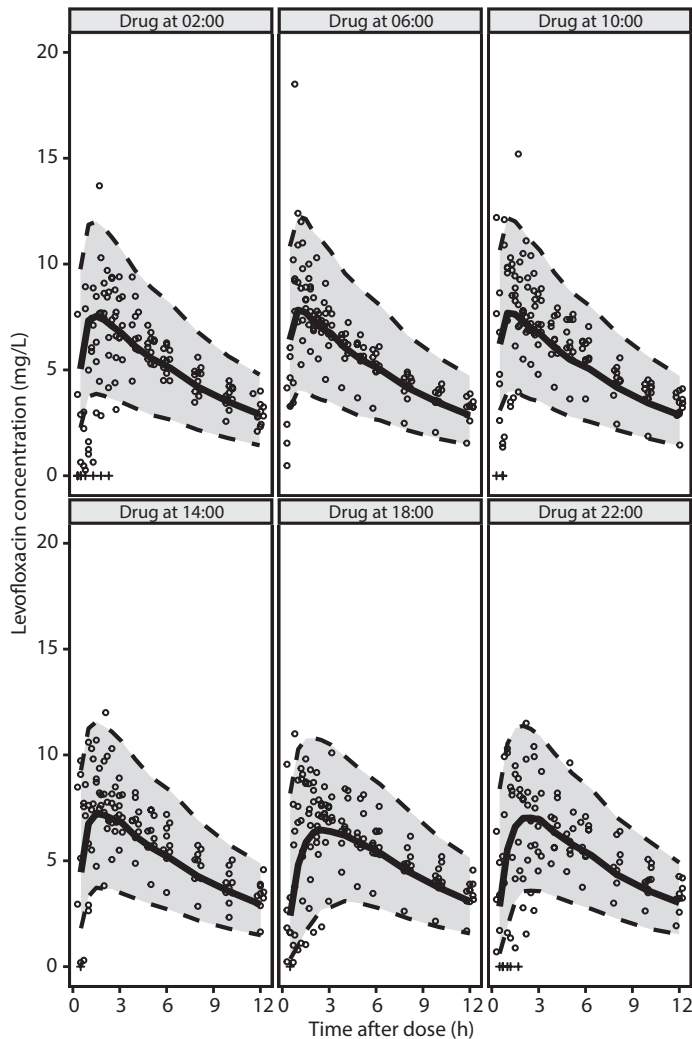

Figure 7 Visual Predictive Check (VPC) stratified by time of administration. Solid line: Median of predicted concentrations, grey area enclosed by dashed lines: 90% prediction intervals of the simulated data. Circles: observed data. Crosses: data points below lower limit of quantification and before Cmax that were included in the data set used for model development.

Table 3 Population parameter estimates of the final PK model.

Parameter estimate		Estimate (RSE %)	IIV %CV
Cl/F (L/h)		10.8 (6%)	18.3%
V/F (L)		116 (4%)	14.1%
	Trough (h^{-1})	2.10 (30%)	91.6%
Ka=Ktr	Amplitude (h^{-1})	1.85 (18%)	ND
	Acrophase (h after midnight)	7.97 (6%)	ND
Residual proportional error (%CV)		29.0%	
Covariance between Cl/F and V/F (%CV)		72.9%	

RSE = relative standard error; CV = coefficient of variation; IIV = inter-individual variability; ND = not determined.

Table 4 Steady-state values of a dosing regimen of once-daily 1000mg oral levofloxacin at 08:00, 18:00 and 23:00 simulated in 500 subjects.

	Time of drug administration		
	08:00	18:00	23:00
$C_{max,ss}$ (mg/L)	8.93 (6.51-12.1)	8.13 (5.51-11.4)	8.53 (6.09-11.7)
$T_{max,ss}$ (h)	1.00 (0.50-1.50)	2.30 (0.70-6.05)	1.75 (0.70-3.25)
AUC_{ss} (h*mg/L)	92.6 (61.4-134)	92.7 (61.3-134)	92.7 (61.4-134)

Median ± 95% confidence intervals. AUC: area under the curve; ss: steady-state

predictive check (VPC) shows that the model describes the observed variability well (Figure 7). Furthermore, non-compartmental analysis (NCA) of the observed profiles and of the model predicted profiles yielded comparable results and largely similar 24-hour fluctuations in Cmax and Tmax (Supplemental Table 1). The median Cmax of the observed data tended to be slightly higher than the median Cmax of the model predicted profiles. One reason for this discrepancy is that the C_{max} (and T_{max}) of the observed data is inherently sensitive to the discrete sampling times employed in a study, while this is less so for a population pharmacokinetic approach. Secondly, the population PK model takes into account residual error in the data, while analysis of the observed data relies on the data points as they are measured.

Simulations

A once-daily dosing regimen of 1000mg oral levofloxacin for seven days was simulated in 500 subjects with dosing times at 08:00, 18:00 and 23:00, representing three typically used dosing times (around breakfast, dinner or bedtime). These simulations show that $T_{max,ss}$, $C_{max,ss}$ and AUC_{ss} are not significantly affected by dosing time (Table 4).

DISCUSSION

In this study, we developed a population pharmacokinetic model based on data from a clinical trial in which levofloxacin was administered to twelve healthy subjects at six different time points throughout the 24-hour period. Levofloxacin pharmacokinetics could

be described by a one-compartment model with first-order clearance and one transit compartment to describe the absorption phase. Ka varied considerably throughout the day and night, which could be parametrized by a cosine function with a fixed period of 24 hours, a mesor of $3.95h^{-1}$, a peak around 8:00 in the morning and a relative amplitude of 47%. This study shows how a chronopharmacological study design can be combined with population pharmacokinetics to quantitate the impact of time of administration on the concentration profiles of a drug.

The parameter estimates reported in the present study are comparable to those from previously published population pharmacokinetic models of oral levofloxacin (Peloquin et al., 2008; Tanigawara et al., 1995; Zhang et al., 2009). In these studies, the population parameter estimates for Ka range from 1.44 to $5.96h^{-1}$. A potential explanation for this wide range of values is that the effect of time of administration was overlooked. We extend the previous findings by showing that the Ka varies over the 24 hour period with population parameter estimates ranging from $2.10h^{-1}$ at 20:00 in the evening to $5.80h^{-1}$ at 08:00 in the morning.

Several mechanisms could underlie the observed 24-hour rhythm in Ka. The rate of absorption of levofloxacin is mainly determined by gastric emptying as levofloxacin has a high solubility and permeability (Chen et al., 2011; Frick et al., 1998; Maezawa et al., 2013). Since gastric emptying time of a solid meal in human subjects shows significant 24-hour variation, being faster at 8:00 (half-time: 64.8±6.4 min) compared to 20:00 (97.1±11.5min) (Goo et al., 1987), the most likely explanation for the finding that the Ka of levofloxacin shows a 24-hour rhythm is variation in gastric emptying. However, we cannot exclude that variation in intestinal blood flow may also play a role (Dallmann et al., 2014).The involvement of other rhythmic processes in the absorption of levofloxacin is likely to be limited. For example, the absorption of levofloxacin is minimally affected by intestinal metabolism(Fish and Chow, 1997). Additionally, rhythmic activity of the efflux transporter p-glycoprotein in the intestine could affect drug absorption (Iwasaki et al., 2015; Okyar et al., 2012). However, the evidence that levofloxacin is a substrate for p-glycoprotein is conflicting (Naruhashi et al., 2001; Yamaguchi et al., 2000, 2001, 2002). Although it has been shown that the intestinal clearance of levofloxacin is reduced in the presence of a p-glycoprotein inhibitor in vivo, plasma concentrations of levofloxacin during the absorption phase were not affected (Yamaguchi et al., 2002).

During all study visits, GFR was measured three hours after levofloxacin administration. It was found that the effect of time of day on GFR was slight but statistically significant, being 9% higher at 09:00 in the morning compared to 01:00 at night. Since levofloxacin is mainly eliminated through passive renal elimination (Chien et al., 1997a, 1997b, 1998; Lubasch et al., 2000; Sprandel et al., 2004) and is only slightly affected by tubular secretion (Chien et al., 1997b; Lubasch et al., 2000; Okazaki et al., 1991; Sprandel et al., 2004), we hypothesized that GFR influences levofloxacin clearance and that the 24-hour variation in GFR is reflected in this parameter. However, including GFR as a covariate on clearance did not significantly improve the fit of the model. Although this observation is in line with some

studies (Peloquin et al., 2008; Tanigawara et al., 1995), other studies did find a correlation between total body clearance of levofloxacin and GFR, as measured by creatinine clearance (Chien et al., 1997b; Chow et al., 2001; Zhang et al., 2009). Possible explanations for this discrepancy are that our homogenous study population had a relatively narrow range of GFR values or that the minimal differences in GFR observed at different time points of administration do not affect the pharmacokinetics of levofloxacin.

Despite the relatively large amplitude of the rhythm in Ka (47%) that has a peak in the morning, the simulations we performed show that the AUC and Cmax, two parameters related to the bactericidal action of levofloxacin (Drusano et al., 2004; Preston et al., 1998; Shams and Evans, 2005), were not significantly influenced by time of administration. Therefore, our results suggest that oral levofloxacin can be dosed without taking into account the dosing time, at least in terms of parameters related to bacterial eradication. However, the rhythm in Ka may be relevant to other drugs that share the same drug disposition characteristics as levofloxacin (high solubility, high permeability and little metabolism) such as chloroquine (malaria prophylaxis and treatment), doxycycline (antimicrobial) and ethambutol (tuberculosis treatment) (Wu and Benet, 2005). Our findings may also apply to drugs with high solubility and high permeability but that are more extensively metabolized and/or have a shorter half-life. This group of drugs contains many drugs with a relative narrow therapeutic index, including CNS-active drugs such as antidepressants, anti-epileptics, and sedatives as well as antivirals and cardiovascular compounds (Wu and Benet, 2005). How different absorption profiles translate to differences in (first-pass) metabolism for these compounds is unknown, but it is conceivable that an increase in the rate or extent of absorption results in higher systemic concentrations.

This prospective study was specifically designed to detect 24-hour variation in pharmacokinetic parameters of levofloxacin. In addition to the use of six time points of administration, our subjects adhered to a stable sleep/wake rhythm with bedtimes between 23:00 and 0:00 and waking times between 07:00 and 08:00 prior to the study visits to ensure that the diurnal variation in physiological processes was not affected by an irregular lifestyle. Because TSH levels in serum show a robust 24-hour rhythm with a peak during the night (Russell et al., 2008; Weeke, 1973), we measured TSH levels hourly during the study visits as a positive control for rhythmic processes. The TSH levels in all our subjects show clear 24-hour variation with the peak at night, indicating that the study design did not interfere with this rhythmic process. Furthermore, the effect of food and fluid was minimized by timing the meals relative to dosing times and by ensuring a constant water intake throughout the day and night. On the one hand, this is a somewhat artificial situation that limits the direct translation of our findings to the clinic. On the other hand, this study increases our understanding of the 24-hour variation in levofloxacin pharmacokinetics in the absence of food effects and can be combined with studies that did investigate these effects (Lee et al., 1997; Tanigawara et al., 1995).

Population pharmacokinetic modelling is a powerful method to identify different sources of variability in pharmacokinetic parameters, such as interindividual variation and the effect

of subject-specific covariates, but it can also be used to explore variation induced by the rhythmic nature of physiological processes (Bienert et al., 2011; Bressolle et al., 1999; Chen et al., 2013; Lee et al., 2014; Musuamba et al., 2009; Salem et al., 2014). Several population pharmacokinetic studies on daily variations in the pharmacokinetics of drugs used the time of drug administration as a covariate (Bienert et al., 2011; Chen et al., 2013; Musuamba et al., 2009; Salem et al., 2014). This approach may be useful when sample collection takes place over a short time-window or when the drug is administered at a few clock times only. However, because levofloxacin has a relatively long half-life (6-8 hours) (Fish and Chow, 1997), we sampled for 12 hours after administration and the different occasions overlap considerably on the 24-hour time scale. In this case, the use of time of administration as a covariate may obscure the 24-hour variation in parameters such as Cl/F and V/F. We circumvented this issue by using the time window during which the samples were taken as a covariate. Applying this approach to Ka, we found that the model fit improved significantly and that the 24-hour variation of this parameter resembled a sinusoidal pattern that could be described instead as a cosine function with a period of 24 hours. The advantage of implementing a cosine function is that it better reflects the continuous nature of the 24-hour variation in physiological processes. Moreover, it enhances the predictive value of the model by providing an estimate of the parameter at all time-points of the 24-hour period.

In conclusion, we show that the Ka of levofloxacin depends on the time of day that can be described by a cosine function with a period of 24 hours, a relative amplitude of 47% and a peak around 8:00 in the morning, while clearance and volume of distribution are not affected by time of day. Our simulations indicate that the 24-hour variation in absorption rate constant does not affect variables related to bacterial eradication such as AUC or Cmax. Therefore, in terms of pharmacokinetics, levofloxacin can be dosed regardless of the time of day. More importantly, these results can be applied to drugs with similar physicochemical properties as levofloxacin. For drugs with a narrower therapeutic index, the rhythm in absorption rate constant may be clinically relevant.

CONFLICT OF INTEREST

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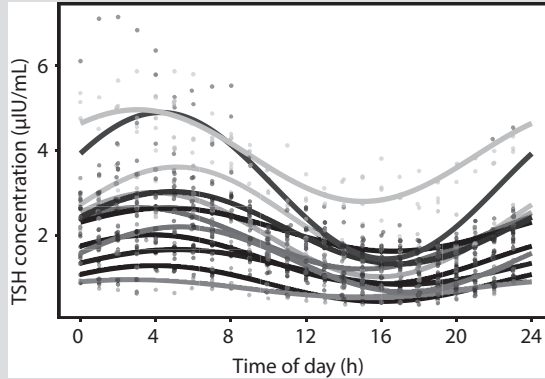
Supplementary Figure 1 Variation in thyroid stimulating hormone (TSH) levels over the course of the 24-hour period in the twelve subjects. Dots: observed data; lines: cosine fitted through the data per subject by cosinor analysis. Different colours represent the 12 different subjects. Data from the six separate occasions were combined.
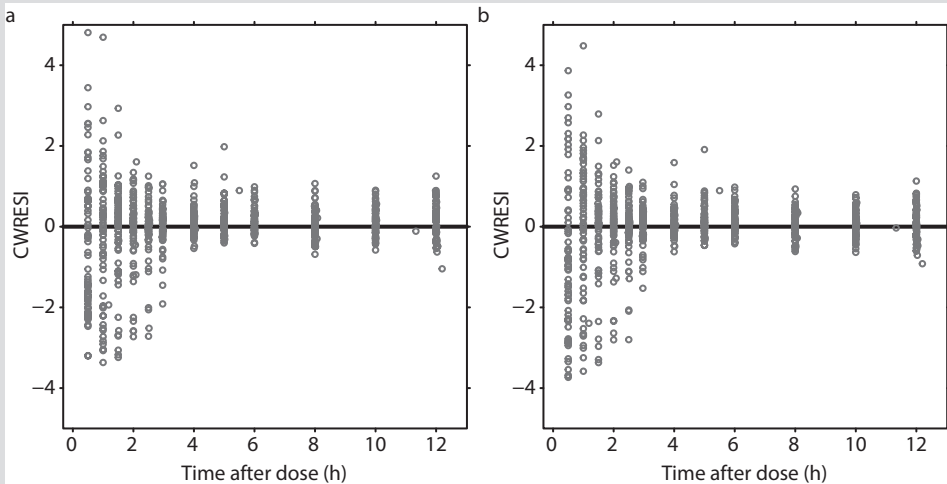
Supplementary Figure 2 Comparison of conditional weighted residuals (CWRESI) versus time after dose of the model without a cosine implemented on Ka **(A)** and of the model with a cosine implemented on Ka **(B)**.

Supplementary Table 1 Comparison of C_{max}, T_{max} and AUC_{0-12} of the observed and individual predicted concentration profiles. Data is shown as median (range).

Time of administration	C_{max} (mg/L)		T_{max} (h)		AUC_{0-12} (h*mg/L)	
	Observed profiles	Individual model predictions[a]	Observed profiles	Individual model predictions[a]	Observed profiles	Individual model predictions[a]
02:00	9.1 (5.5-14)	8.1 (5.6-9.4)	2.0 (1.0-5.0)	1.2 (0.8-1.9)	59.9 (33.3-79.3)	63.3 (40.9-72.9)
06:00	9.3 (7.8-19)	8.3 (5.8-9.7)	1.0 (1.0-2.0)	0.9 (0.7-1.4)	64.2 (39.6-75.0)	63.8 (41.1-73.8)
10:00	10 (6.9-15)	8.3 (5.7-9.6)	1.0 (0.5-2.5)	1.0 (0.7-1.5)	66.5 (42.6-74.7)	63.7 (41.1-73.7)
14:00	9.2 (5.5-12)	7.8 (5.5-8.8)	1.3 (0.5-3.0)	1.3 (0.9-2.4)	64.7 (38.4-79.3)	62.6 (40.7-72.2)
18:00	8.7 (6.7-11)	7.4 (5.1-8.5)	1.8 (1.0-4.0)	1.9 (1.1-6.5)	63.2 (41.0-76.7)	61.3 (39.9-68.4)
22:00	9.8 (5.7-12)	8.0 (5.3-8.7)	2.3 (1.0-5.5)	1.6 (1.0-3.7)	63.7 (42.2-74.9)	63.8 (40.2-69.9)

C_{max}: maximal concentration; T_{max}: time to reach C_{max}; AUC_{0-12}: area under the concentration-time curve from 0 to 12h after administration.