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PHARMACODYNAMICS

Mixed-effect circadian rhythm model for human erythrocyte acetylcholinesterase activity—application to the proof of concept of cholinesterase inhibition by acorn extract in healthy subjects with galantamine as positive control

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Abstract
Purpose The aim of this study was to develop a non-linear mixed effect circadian rhythm model of acetylcholinesterase (AChE) activity variation and to evaluate the inhibitory effect of acorn extract (2 g) and galantamine (16 mg), used as positive control, on human AChE in red blood cells (RBC).
Methods This was an open-label, randomized, three-way crossover study involving 12 healthy subjects who received one of the treatments in each study period: no treatment, acorn extract, and galantamine. RBC AChE activity was measured in peripheral blood samples collected at 0 (pre-dose), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16 and 24 h post-dose administration. Non-linear mixed effect modeling was performed using NONMEM (ver. 7.0).
Results The circadian variation of AChE activity was best described using two mixed effect cosine functions, with periods of 24 and 12 h, respectively. When the inhibitory effect terms were added, the model was significantly improved for both acorn extract and galantamine. In terms of the effect, a 2-g single dose of acorn extract showed AChE inhibition (about 5%) similar to that of a 16-mg single dose of galantamine, in the first 24 h after administration.
Conclusions Based on the very pronounced inter- and intra-day variation in AChE activity in RBC, we conclude that the model-based approach is essential for the proof of concept and quantitation of AChE inhibition in human subjects.

Keywords Mixed effect modeling · Acetylcholinesterase · Acorn extract · Healthy subjects · Proof of concept

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Introduction

Alzheimer-type dementia is a major senile disease which mainly affects cognitive function and results in a deterioration of life quality [1]. Acetylcholinesterase (AChE) activity, which is considered to be one of the etiologic factors for Alzheimer's disease, has been studied extensively [2–4]. Drugs targeting the inhibition of AChE activity, such as donepezil and galantamine, have been developed for symptomatic treatment. Recently, an animal study showed that oral administration of acorn (*Quercus acutissima Carr.*) crude extract had significant inhibitory effect on AChE activity in the mouse brain tissue, with an increase of acetylcholine level [5]. Based on this evidence, a healthy subject trial was carried out to investigate whether the extract had an inhibitory effect on human AChE in vivo. The circadian variation of AChE activity in each subject was an important consideration when this clinical study was being designed. Although AChE circadian variation is one of well-known enzymatic characteristics in humans [6], a quantitative model for the time course of the fluctuation of AChE activity has not yet been published. We report here our development of a non-linear, mixed

effect circadian rhythm model of variations in AChE activity in healthy subjects. This model was used to evaluate the inhibitory effect of a single 2-g dose of acorn crude extract and galantamine (16 mg; positive control), on human AChE in red blood cells (RBC). The cell line was chosen for its excellent accessibility as well as the applicability as a surrogate marker for studies on nerve agents [7].

Subjects and methods

Inclusion and exclusion criteria

Healthy male volunteers, aged 20–45 years, with no clinically relevant conditions identified based on their medical history, physical examination, laboratory tests, and electrocardiography (ECG), were eligible for inclusion. Subjects with any medical history that could result in the alteration of either AChE activity or cholinergic regulation were excluded. The final study population comprised 12 individuals.

Study design

An open-label, randomized, three-way crossover clinical study was conducted at a single center (Clinical Trial Center, St.Mary's Hospital, Seoul). The study was designed using the William's square design, including three 3-day treatment periods with 1-week washout intervals. The sequences at each period were, as follows: ABC, ACB, BAC, BCA, CAB, CBA (A: no treatment; B: acorn extract; C: galantamine). All volunteers were equally allocated to each study sequence at enrollment. During period A, nothing was given to the subjects, but serial blood sampling was performed to measure AChE activity fluctuation at pre-planned time points, starting from dosing time (0800 hours). During periods B or C, 2 g of acorn, *Quercus acutissima Carr.*, extract (B) or 16 mg of galantamine (C) was given with 240 mL purified water, and blood sampling was performed following the same scheme as in period A.

The trial was designed and monitored in accordance with the good clinical practice guidelines of Korea and the principles of the Declaration of Helsinki. The independent institutional review board (Seoul St. Mary's Hospital) approved the protocol prior to the initiation of the trial, and all participants provided written informed consent.

Study procedures

Subject control

All of the subjects were hospitalized from the day before the dosing until the planned blood sampling was completed during each study period. Irrespective of treatment, no food was allowed from 1000 hours on the day of admission to 4 h after dosing time. Drinking of purified water was also prohibited for 2 h before and after dosing. Subjects were also to abstain from taking medications other than the study drugs, drinking alcohol, or engaging in unusual physical exercise from 3 days before each dosing day.

Blood sampling

Whole-blood samples for the determination of AChE activity in RBC were obtained, and safety was monitored throughout the study. The samples, consisting of 8 mL peripheral venous blood, were collected into heparinized tubes at pre-dose and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, and 24 h post-dosing and immediately cooled in an ice bath. A 100-uL sample of whole blood from each blood sample was then mixed with 15 mL phosphate buffer (0.1 mol/L, pH 7.4) and stored in microtubes (1.5 mL each) at −20°C, until assay.

Acetylcholinesterase activity determination

Whole-blood samples were analyzed using a spectrophotometer (DU530; Beckman Coulter, Brea, CA) according to the method of Worek et al. (1999) [8]. The thawed samples were kept on ice until analysis. AChE activities were measured at 436 nm and 37ºC. For the determination of total hemoglobin, the absorption at 546 nm was then read against a water blank ($\varepsilon = 10.8 \times 10^{-3}\ M^{-1}cm^{-1}$). The hemoglobin concentration (μmol/L), enzyme activity, and the specific activity of erythrocyte AChE were calculated using the following equations:

$$\text{Hemoglobin}\ (\mu mol/L) = Absorption(A) \times 1000/10.8$$

$$\text{Activity}\ (\mu mol/L/\min) = \frac{Sample(mE/\min) - Blank(mE/\min)}{10.6}$$

$$\text{AChE}\ (mU/\mu mol\,Hb) = \frac{Activity(\mu mol/L/\min) \times 1.58 \times 1000}{Hb(\mu mol/L)}$$

The assay linearity of AChE activity in different dilutions of the AChE enzyme was acceptable ($r^2 = 0.9994$). Intra-day accuracy (106.8%) and precision [coefficient of variance (CV) 5.1%], and inter-day accuracy (102.9%) and precision (CV 5.3%) were acceptable. To ensure the validity of the measurements and to minimize the influence of analytical conditions on the results, all samples from an individual were analyzed in the same batch, and control samples were measured repeatedly in

every five samples. The results were accepted only when the precision of the control sample measurements was <10% (CV %).

Model development for the circadian rhythm of AChE and its inhibition

A mixed effect analysis was conducted using NONMEM (ver. 7.0; Icon Development Solution, Ellicott City, MD).

Dataset

The dataset consisted of a total of 525 observations from 12 subjects (45 observations/subject; 1 subject left the study during the last period). The data collected included the measurements of AChE activity, as well as the demographic characteristics of the participants, such as weight, height, age, and sex.

Structural model building

Although the circadian rhythm of AChE activity in humans has been extensively reported, the underlying physiological mechanism is still unclear. Due to this lack of mechanistic understanding, we have constructed an empirical model for circadian rhythm using the multiple components procedure for analysis of longitudinal time-series amounts, as reported by Fernandez and Hermida (1998) [9], which can be widely used to explain periodic rhythm.

$$\text{AChE activity} = MESOR + \sum_{j=1}^{m} AM_j \times \cos\left\{ (TIME - AC_j) \times \frac{2\pi}{PER_j} \right\}$$

where MESOR is the midline estimating the statistic of rhythm, AM_j is the amplitude of variation produced by the *j*th component of variation, AC_j is the acrophase of the *j*th component of variation, and PER_j is the period of the *j*th component of variation (assumed to be divisors of 24 h). Inter-occasional variability (IOV) for MESOR was allowed, because there was considerable variation of MESOR between periods in the same subject. The components were added up one by one, from the one with the longest period (in the order of 24, 12, 6 h, etc.) until the model could not be improved any more. After the circadian model had been constructed, the inhibitory effect was subtracted (i.e., added as a negative quantity). Because neither serum galantamine concentration nor any active component of the acorn extract was measured, we assumed that the time course of inhibitory effects was identical to that of drug concentrations following first-order absorption and elimination after dosing. The formation rate constants (KA_{acorn}, $KA_{galatamine}$, in place of the absorption rate constant) and the elimination rate constants (KE_{acorn}, $KE_{galatamine}$) of the effects, in addition to doses and scaling parameters (S_{acorn}, $S_{galatamine}$), were used to formulate the equation for the time–effect relationship, as follows:

$$\text{Effect} = \left(\frac{Dose}{S}\right) \bullet \left(\frac{KA}{KA - KE}\right) \bullet \left(e^{-KE \bullet TAD} - e^{-KA \bullet TAD}\right)$$

where TAD represents the elapsed time after dosing.

Use of between-subject variability (BSV, η_I) values to find individual parameters (P_I) in relation to the population parameters (TVP) were tested in the model-building process, where the BSV (η_I) for each parameter followed a Gaussian distribution with a mean of 0 and differing values of variance (described using the symbol ω^2). The combined (i.e., additive and proportional) error model was initially applied to describe the residual error, including the intra-individual variability and the measurement error, as follows:

$$DV_{ij} = DV_{ipred,ij} \bullet \left(1 + \varepsilon_{\text{prop},ij}\right) + \varepsilon_{\text{add},ij}$$

where DV_{ij} is the *j*th measured AChE activity in the *I*th individual, $DV_{ipred,ij}$ is the *j*th predicted value in the *I*th individual, and $\varepsilon_{prop,ij}$ and $\varepsilon_{add,ij}$ are the residual variability with a mean of 0 and a variance of σ_{prop}^2 and σ_{add}^2, respectively. The correlation between the random variables in the model was explored using the $COVARIANCE block. The first-order conditional estimation (FOCE) approximation method, with $\eta - \varepsilon$ interaction, was chosen for the estimation process.

The appropriateness of the models was comprehensively evaluated based upon various goodness-of-fit criteria, including visual comparison of diagnostic scatter plots, likelihood ratio tests (LRT), measures of model stability or adequacy (i.e., condition number, successful convergence, significant digits, matrix singularity), among others. The results for LRT were considered to be statistically significant if decreases in the objective function value (OFV) were greater than the cut-off points equivalent to the *p* value 0.05 (i.e., 3.84 for $df = 1$; 5.99 for $df = 2$, etc.). After the basic model had been constructed, the subjects' demographics included in the dataset were screened as potential covariates for the investigated parameters.

Model evaluation

To evaluate the parameter values obtained from the final model, 1,000 bootstrap-resampled datasets from the original dataset were sequentially estimated using the same final model. The median and 95% confidence intervals (CI, 2.5 to 97.5 percentile) of parameters from this step were compared with the final parameter estimates. A visual-predictive check (VPC) was also performed. A total of

54,000 data points from 1,000 virtual subjects (i.e., following the same 3-way crossover design as the actual study, with 18 observations per period) were simulated, using the final model for circadian rhythm and the inhibitory effect. With this simulated data, the 75% CI of AChE activity at each observation time were overlaid with the observed raw time–activity data.

Results

The mixed effect model for circadian rhythm

The BSV for individual parameters describing the circadian rhythm was explained best when it was applied additively (e.g., + η_I). The final population parameter estimate for MESOR was 520 mU/μmol. The model was significantly improved when about 20% (CV) of BSV and IOV was applied to MESOR. The equations for BSV and IOV are as follows:

$$\text{Individual MESOR at Each Period} = \theta_{MESOR} + \eta_{MESOR} + IOV$$

$$IOV = PER_2 \times \eta_{PER2} + PER_3 \times \eta_{PER3}$$

where θ_{MESOR} is the typical value of MESOR in the population, η_{MESOR} is the BSV determined at the first study period. PER_2 is an indicator variable which has value of 0 at any time other than the second period where PER_3 is the same variable for the third period. η_{PER2} *and* η_{PER3} is the IOV at according period. The variances of η_{PER2} *and* η_{PER3} was assumed to be identical.

The circadian variation in AChE activity was best described with two cosine functions with periods of 24 and 12 h, respectively. The 24-h component had 11.0 mU/μmol amplitude and a 20.8-h acrophase where those of the 12-h component were 3.05 mU/μmol and 10.6 h, respectively. The BSV was significant at the amplitude parameters only. No covariance structure or contributing covariates were identified.

The population model for inhibitory effect

When the inhibitory effect part was added to the circadian model, the estimation results were significantly improved for both the acorn extract and galantamine, which clearly demonstrated the inhibition of AChE activity by both treatments. The goodness-of-fit plot of the final model is given in Fig. 1 (see Appendix for more data). The BSV for individual parameters describing inhibitory effect was applied exponentially [i.e., × exp(η_I)], and the BSV terms were identified at the formation rate constant for both of treatments. No covariance structure or contributing covariates were identified. The overall process of model development is summarized in Table 1.

Model evaluation and simulation

The parameter estimates and their 95% CI obtained from the bootstrap process are given in Table 2. The VPC results in Fig. 2 shown as 75% simulated CI range curves overlaid with the observed data (i.e., no treatment vs. either galantamine or acorn extract) demonstrate that both treatments had significant inhibitory effects. For the first 24 h after a single oral dose, the degree of inhibitory effect by 2 g acorn extract was found to be similar to that exerted by 16 mg galantamine used as the positive control.

Discussion

In this study, we evaluated the inhibitory effect of the acorn extract on human AChE activity, compared to that of galantamine, by constructing a mixed effect model for the circadian rhythm and drug effect. The circadian model adequately described the intra-day variation in AChE activity. Although no drug concentrations were measured, the inhibitory effect model developed under the assumption that the magnitude of the effect follows first-order kinetics appeared to be useful for separating the inhibitory effect component from the circadian variation.

The building of a circadian rhythm model was an essential part of this analysis. When overall AChE activity, i.e., the area under the time–concentration curve (AUC) of AChE activity for each period was compared using conventional statistical tests (Student *t* test for calculated AUC of AChE activity between the A and either B or C treatment), the significance of the inhibitory effect could not be demonstrated, even for the positive control (galantamine), because of the wide fluctuation in AChE activity. Only after modeling the circadian components of AChE activity, were we able to identify the inhibitory effects as a part of the model. Our model reflected well the pattern of intra-day change in AChE activity and identified BSV and residual errors included in the raw data. Using the model, we were able to establish a standard for comparison when evaluating the effects during the treatment periods. With regard to the inhibitory effect, in the first 24 h after administration, a 2-g single dose of acorn extract showed AChE inhibition (about 5%) similar to that of a 16-mg single dose of galantamine.

There are a number of limitations in the extrapolation using our model. The exact dose–response relationship could not be established because of the single-dose design in our healthy subjects study. Although we could only

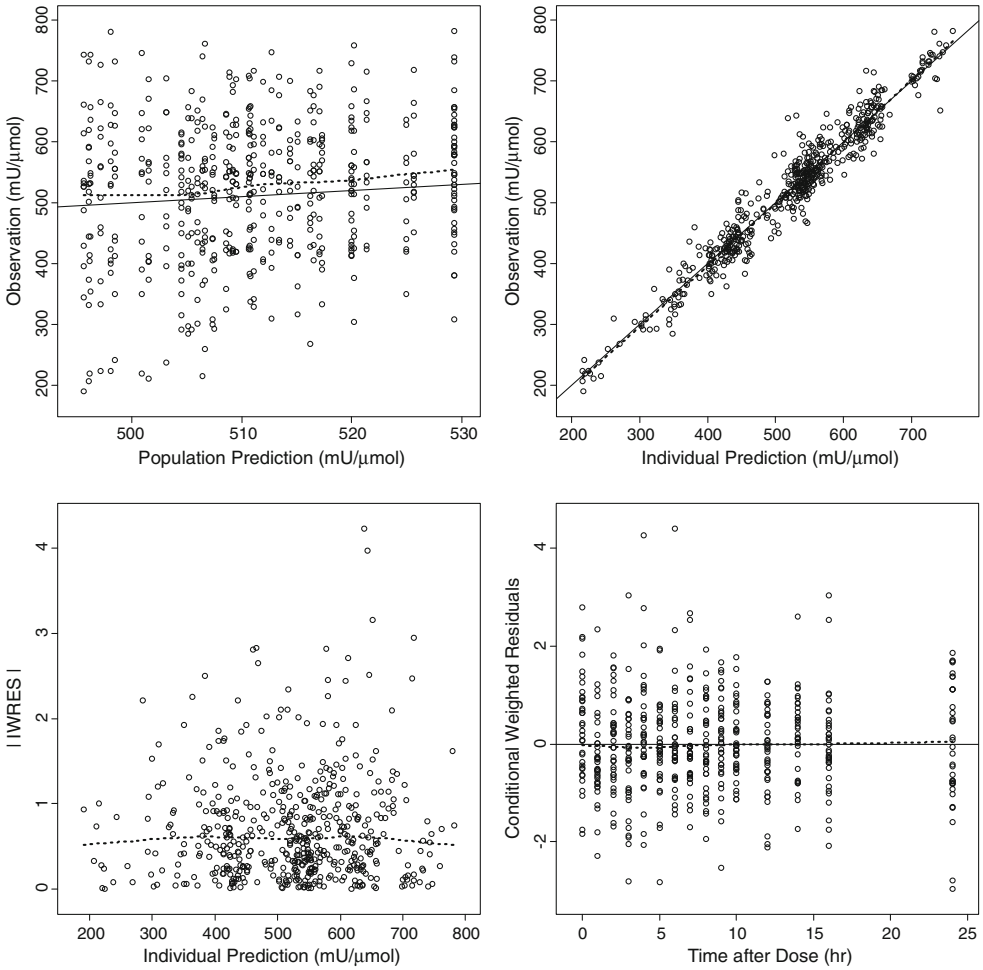

Fig. 1 Basic goodness-of-fit plot. *Solid line* Line of identity (**upper panels**), line of reference ($y = 0$, **bottom right panel**). *Dotted line* Line of locally weighted scatterplot smoothing (LOWESS)

assume a simple linear dose–response relationship, it may have been a saturable type of relationship (e.g., Emax or something else), which can be estimated only after trials with several different dosage regimens. In addition, this study was an exploration of the inhibitory effect of acorn extract on AChE activity in humans—and not in patients with dementia. The model for circadian rhythm and drug effect presented in this report might be applicable to proof-of-concept studies of pharmacologic agents targeting AchE inhibition; however, possible differences in AchE activity according to the site of action (e.g., RBC surface or central nervous system) or patient characteristics, such as age or disease status, were not considered. Another concern is that the duration of observation (i.e., 24 h) was found to be too short to describe the full time-course of the effect. The inhibitory effects lasted for more than 24 h for both acorn extract and galantamine, as shown in Fig. 2. This resulted in the wide confidence intervals of the elimination parameters and in an impossibility to estimate the BSVs for these. Therefore, either dose escalation or multiple dosing studies, with periods of observation much longer than 24 h, seem to be necessary to obtain a better model.

In conclusion, we successfully demonstrated AChE inhibition by both acorn extract and galantamine in

Table 1 Model development process

Model development	OFV	ΔP	p =0.05	ΔOFV
Baseline only	4349.330	–	–	–
Addition of 24-h period variation	4301.856	3	−7.81	−47.474
Addition of 12-h period variation	4286.627	3	−7.81	−15.229
Addition of acorn extract effect	4275.324	4	−9.49	−11.303
Addition of galantamine effect	4261.596	4	−9.49	−13.728

OFV, Objective function value; ΔP, number of parameters added at each step; ΔOFV, observed OFV changes

Table 2 Final model parameter estimates and evaluation results

Parameter	Estimate	Bootstrap result	
		Median	95% Confidence interval[a]
Fixed effect			
MESOR (mU/μmol)	520	518	466 ~ 574
AM24 (mU/μmol)	11.0	11.8	6.79~18.0
AM12 (mU/μmol)	3.05	3.25	0.0320~8.96
AC24 (h)	20.8	20.9	19.1~23.9
AC12 (h)	10.6	10.6	9.81~12.0
S_{Acorn} (mg · μmol/mU)	0.816	0.999	0.0900~19.9
KE_{Acorn} (h^{-1})	0.492	0.422	0.0450~19.5
KA_{Acorn} (h^{-1})	0.00279	0.00383	0.000410~0.151
$S_{Galantamine}$ (mg · μmol/mU)	0.108	0.113	0.0914~0.426
$KE_{Galantamine}$ (h^{-1})	0.023	0.0189	0.000311~0.0348
$KA_{Galantamine}$ (h^{-1})	0.00465	0.00546	0.000255~0.0240
Between-Subject variability (CV %)			
MESOR during the 1st period	20.4	19.2	12.7~25.4
IOV during the 2nd and 3rd period	20.5	21.3	10.4~29.9
AM24	51.1	48.5	5.14~85.0
AM12	311	305	152~403
KA_{Acorn}	143	122	10.6~217
$KA_{Galantamine}$	398	407	157~819
Residual error			
σ_{add} (mU/μmol)	28.4	27.7	23.9~31.9

MESOR, Midline estimating the statistic of rhythm, AM, amplitude of variation; AC acrophase; S, scaling parameter; KA, formation rate constant; KE, elimination rate constant; IOV, inter-occasional variability

[a]95% Confidence intervals obtained from the estimation of 1,000 bootstrap-resampled datasets

healthy subjects using the models described in this report. Our circadian model for AChE activity may be further applied to the proof of concept for AChE inhibition in early phase clinical trials of other new chemical entities. Our results also exemplify the usefulness of modeling and simulation approaches for detecting the effect(s) of drugs on biomarkers showing wide circadian variation.

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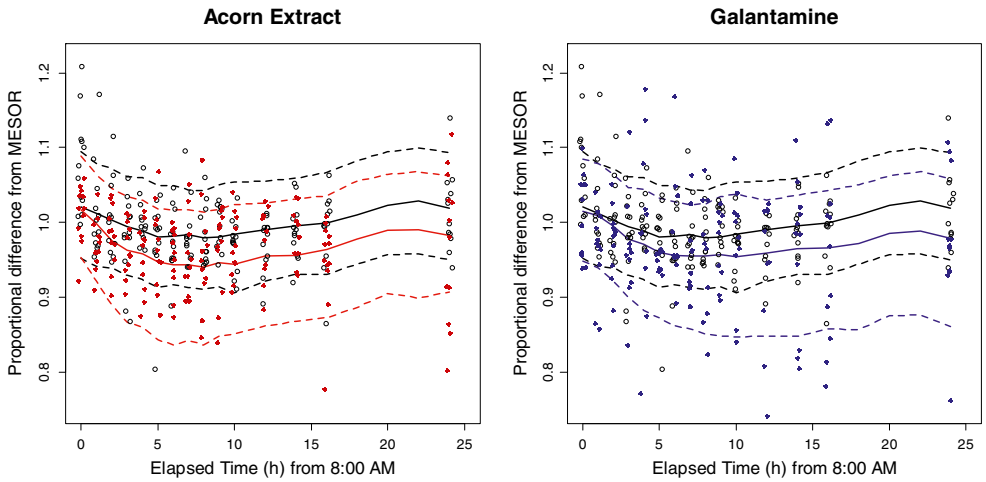

Fig. 2 Visual predicted checks using observed and simulated acetylcholinesterase (AChE) activity. *Circles* Observed AChE activity data (*open* no-treatment period, *red* acorn extract, *blue* galantamine); these AChE activity symbols were jittered to avoid overlapping between treatment and no-treatment periods. *Black lines* Median (*solid*) and 75% confidence interval (CI; *broken*) of simulated AChE activity for the no-treatment period. *Colored lines* median (*solid*) and 75% CI (*broken*) of simulated AChE activity with the respective treatment (*red* acorn extract, *blue* galantamine). *MESOR* Midline estimating the statistic of rhythm

Appendix

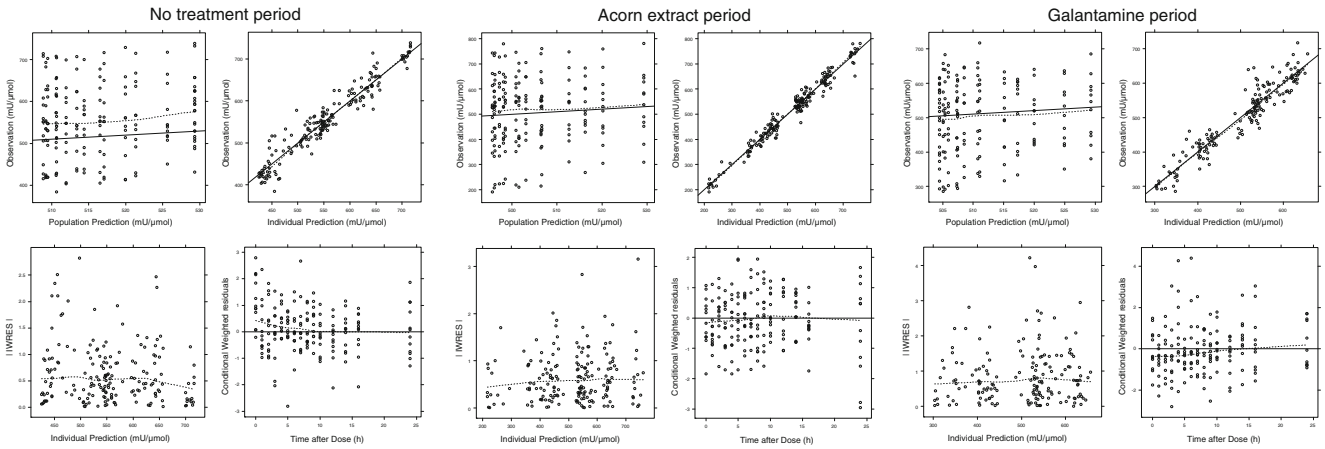

Fig. 3 Basic goodness-of-fit plot for each treatment period. *Solid line* Line of Identity (**upper panels**), line of reference (y=0, **bottom right panel**). *Dotted line* Line of locally weighted scatterplot smoothing (LOWESS)

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