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DOSE-DEPENDENT EEG EFFECTS OF ZOLPIDEM PROVIDE EVIDENCE FOR $GABA_A$ RECEPTOR SUBTYPE SELECTIVITY *IN VIVO*.

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Non-standard abbreviations used: PK/PD modeling: pharmacokinetic-pharmacodynamic modeling. EEG: electroencephalogram. HPLC: high performance liquid chromatography. *Cl*: total body clearance. Q_2, Q_3: inter-compartmental clearance 2 and 3. V_1, V_2, V_3: volumes of distribution of compartment 1, 2 and 3. E_0: baseline EEG. E_{top}: maximal EEG effect. e_{PD}: *in vivo* relative efficacy. K_{PD}*: in vivo* drug potency. *a*: coefficient determining steepness of parabola. *b*: stimulus intensity where the top of the parabola is reached. *d*: exponent determining the asymmetry of the parabola, *A*: constant which amplifies variation in baseline into parameter *a*.

ABSTRACT

Zolpidem is a non-benzodiazepine $GABA_A$ receptor modulator which binds *in vitro* with high affinity to $GABA_A$ receptors expressing α_1 subunits but with relatively low affinity to receptors expressing α_2, α_3 and α_5 subunits. In the present study it was investigated whether this subtype selectivity could be detected and quantified *in vivo*. Three doses (1.25, 5 and 25 mg) of zolpidem were administered to rats in an intravenous infusion over 5 min. The time-course of the plasma concentrations was determined in conjunction with the change in the β-frequency range of the EEG as pharmacodynamic endpoint. The concentration-effect relationship of the three doses showed a dose-dependent maximum effect and a dose-dependent potency. The data were analyzed for one- or two-site binding using two pharmacodynamic models based on i) the descriptive model and ii) a novel mechanism-based PK/PD model for $GABA_A$ receptor modulators that aims to separates drug- and system-specific properties, thereby allowing the estimation of *in vivo* affinity and efficacy. The application of two-site models significantly improved the fits compared to one-site models. Furthermore, in contrast to the descriptive model, the mechanism-based PK/PD model yielded dose-independent estimates for affinity (97 ± 40 and 33,100 ± 14,800 $ng \cdot ml^{-1}$). In conclusion, the mechanism-based PK/PD model is able to describe and explain the observed dose-dependent EEG effects of zolpidem and suggests the subtype selectivity of zolpidem *in vivo*.

The $GABA_A$ receptor is a hetero-oligomeric protein consisting of five subunits that form an integral Cl^- channel (see for review Sieghart, 1995). To date, various $GABA_A$ receptor subunits and their isoforms (α_1-α_6, β_1-β_3, γ_1-γ_3, δ, θ, ε, π, and ρ) have been described (Barnard *et al.*, 1998; Sieghart, 2000). In theory, these subunits can assemble to many $GABA_A$ receptor subtypes. In the central nervous system, however, functional $GABA_A$ receptors are formed mainly by combinations of α, β and γ subunits (Barnard, *et al.*, 1998).

According to a historical classification, benzodiazepines exert their anxiolytic/hypnotic actions through the activation of two pharmacologically distinct binding sites, ω_1 (BZ_1) and ω_2 (BZ_2), which were classified on the basis of differing affinities of CL 218.872 and zolpidem, respectively. In the mean time, it has been shown in *in vitro* investigations that zolpidem, which is a hypnotic of the imidazopyridine class, differs from conventional benzodiazepines (i.e. flunitrazepam, diazepam) and other hypnotics (zopiclone). Zolpidem displays high affinity at $GABA_A$ receptors expressing α_1 subunits (K_i = 15-350 nM) but a relatively low affinity at receptors expressing α_2, α_3 and α_5 subunits (K_i = 4–40 µM), whereas benzodiazepines have equal affinity for the various $GABA_A$ receptor subtypes (Benavides *et al.*, 1993; Luddens and Korpi, 1995; Pritchett *et al.*, 1989; Ruano *et al.*, 1992). In addition, it has become clear that receptors with α_1 subunits mediate sedative and hypnotic effects, whereas receptors consisting of α_2, α_3 and α_5 subunits are involved in mediating anxiolytic and anticonvulsant effects (Barnard, *et al.*, 1998; Perrault *et al.*, 1988).

The knowledge that specific pharmacological effects are mediated by a specific subunit composition may stimulate the development of drugs with a higher subtype selectivity, resulting in selective clinical effects (Sieghart, 2000). The rational design of drugs with affinity to specific receptor subtypes has become possible and is mainly based on the progress of research using biochemical techniques in isolated systems and knockout strategies (Sieghart, 2000; Mohler *et*

al., 2002). However, the dissection of pharmacological effects mediated by various receptor subtypes requires also detailed investigations *in vivo*. Recently, Rowlett *et al.*, (2000) have provided evidence for the involvement of α_1 subtypes of $GABA_A$ receptors in the transduction of the discriminatory stimulus effects of zolpidem at higher doses, compared to the classical benzodiazepine diazepam *in vivo*. Furthermore, it has been demonstrated in mice that the sedative-hypnotic and anti-convulsant activities of zolpidem are due to its action at α_1 but not at α_2 or α_3 $GABA_A$ receptor subtypes (Crestani *et al.*, 2000). So far, however, little progress has been made in the analysis of the pharmacokinetic-pharmacodynamic relationship of zolpidem with respect to its subtype selectivity.

Recently, a mechanism-based PK/PD model has been developed for the EEG effects of a wide array of $GABA_A$ receptor modulators including neuroactive steroids, benzodiazepines, imidazopyridines, cyclopyrrolones and β-carbolines (Visser *et al.*, 2002a; Visser *et al.*, 2002b; Visser *et al.*, 2003). This model comprises a separate characterization of i) the receptor activation process and ii) the signal transduction process. In this model the receptor activation process is described by a hyperbolic function, while the signal transduction process is described by a parabolic transducer function. It has been demonstrated that on the basis of this model estimates of the *in vivo* affinity and intrinsic efficacy can be obtained, which are closely correlated to estimates obtained in *in vitro* bio-assays, confirming the validity of the model.

An intriguing question is whether this newly developed mechanism-based PK/PD model is able to account for the activation of different $GABA_A$ receptor subtypes. Therefore, the aim of the present investigation was to investigate the subtype selectivity of zolpidem *in vivo*. To this end, increasing doses of zolpidem were administered to rats. The zolpidem concentration-effect relationships were analyzed for one-site and two-site binding using a descriptive model and the novel mechanism-based PK/PD model for $GABA_A$ receptor modulators.

METHODS

Animals and surgical procedures

The protocol of this investigation was approved by the Ethical Committee on Animal Experimentation of Leiden University. Male Wistar rats (305 ± 19 g (mean ± SD), Broekman Breeding Facilities, Someren, The Netherlands) were used. Following surgery, the rats were housed individually in standard plastic cages with a normal 12-hour day/night schedule (lights on 7 AM) at a temperature of 21°C. The animals had access to standard laboratory chow (RMH-TM, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum.*

Nine days before the start of the experiments seven cortical electrodes were implanted into the skull at the locations 11 mm anterior and 2.5 mm lateral (F_l and F_r), 3 mm anterior and 3.5 mm lateral (C_l and C_r) and 3 mm posterior and 2.5 mm lateral (O_l and O_r) to lambda, where a reference electrode was placed (see Visser *et al.*, 2002a). Stainless steel screws were used as electrodes and connected to a miniature connector, which was insulated and fixed to the skull with dental acrylic cement.

Three days before the start of the experiment, indwelling cannulae were implanted in the right femoral artery for the serial collection of blood samples and in the right jugular vein for drug administration. The cannulae, filled with heparinized 25% PVP solution, were tunneled subcutaneously to the back of the neck where they were exteriorized and fixed with a rubber ring. The surgical procedures were performed under anesthesia with 0.1 $mg \cdot kg^{-1}$ i.m. of medetomidine hydrochloride (Domitor, Pfizer, Capelle a/d IJssel, The Netherlands) and 1 $mg \cdot kg^{-1}$ s.c. of ketamine base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands). After the first surgery, 4 mg of ampicilline (A.U.V., Cuijk, The Netherlands) was administered to aid recovery.

Treatment and dosages

Zolpidem was obtained from Sigma Alldrich BV (Zwijndrecht, The Netherlands). Infusion solutions of zolpidem were prepared in 250 µl saline with equimolar hydrochloric acid. Rats were randomly assigned to treatment groups (n=8/8/8/8, based on power calculations) that received vehicle, 1.25, 5 or 25 mg of zolpidem in a 5 min zero-order infusion, which corresponded to a dose of 4.0 ± 0.1, 16.7 ± 0.4 and 83.1 ± 1.1 $mg \cdot kg^{-1}$, respectively.

In vivo pharmacological experiments

The studies were conducted in accordance with the requirements of national legislation and appropriate guidelines for animal care. All experiments were started between 8.30 and 9.30 AM to exclude influences of circadian rhythms. The rats were placed in a rotating drum to control the level of vigilance, thereby avoiding the interference of sleep patterns. During the experiments, the rats were deprived of food and water for the duration of the experiment (max 450 min). Bipolar EEG leads on the left hemisphere (F_l-C_l) were continuously recorded using a Nihon-Kohden AB-621G Bioelectric Amplifier (Hoekloos BV, Amsterdam, The Netherlands) and concurrently digitized at a rate of 256 Hz using a CED 1401_{plus} interface (CED, Cambridge, UK). The signal was fed into a 80486 computer (Intel BV, Sassenheim, The Netherlands) and stored on hard-disk for off-line analysis. After recording of the EEG baseline for 45 min, a 5 min zero-order intravenous infusion of zolpidem was administered to the conscious and freely moving rats using an infusion pump (Bioanalytical Systems Inc., Indiana, USA). For each 5 sec epoch, quantitative EEG parameters were obtained off-line by Fast Fourier Transformation with a user-written script within the data analysis software package Spike 2, version 4.6 (CED, Cambridge, UK). Amplitudes in the β-frequency band of the EEG (11.5-30 Hz), averaged over 1 min time intervals, were used as a measure of drug effect intensity.

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Serial arterial blood samples were taken at pre-defined time-points and the total volume of blood samples was kept equal to 1.8 ml during each experiment. The blood samples were heparinized and centrifuged at 5000 r.p.m. for 15 min for plasma collection. The plasma samples were stored at –20°C until high performance liquid chromatographic (HPLC) analysis.

HPLC analysis

The plasma concentrations of zolpidem were determined by a specific HPLC assay with UV detection as described previously (Visser *et al.,* 2003). Briefly, the samples were diluted with 0.5 ml 0.1 M NaOH, 50 µl of clobazam as internal standard (1 µg/ml in MeOH) was added and the mixture was extracted with 5 ml dichloromethane/petroleum ether (45:55, v:v). The mixture was vortexed for 5 min and subsequently centrifuged for 15 min at 5000 r.p.m. The samples were placed at –20°C to freeze the waterphase. The organic phase was transferred to a clean tube and evaporated under reduced pressure at 37°C. The residue was dissolved in 150 µl of mobile phase of which 40 µl was injected into the HPLC system. A mixture of 25 mM phosphate buffer and acetonitrile (60:40 v:v, pH 7.0) was used as mobile phase for zolpidem. The chromatographic system consisted of a M-45 solvent delivery pump, a WISP 717 automatic injector (all of Millipore-Waters, Milford, USA), a 150 x 4.6 mm C18 5µ column (Alltech BV, Breda, The Netherlands) equipped with a hand-packed C18 guard column (20 mm x 2 mm I.D.) and a spectroflow 757 Kratos UV detector (Spark Holland BV, Emmen, The Netherlands). Zolpidem was detected at 215 nm. The detector-output was recorded using a Shimadzu C-R3A integrator (Shimadzu, ‘s Hertogenbosch, The Netherlands). Linear calibration curves were obtained in the range of 0.05-10 $\mu g \cdot ml^{-1}$ for the 4 $mg \cdot kg^{-1}$ dose, in the range of 0.05-50 $\mu g \cdot ml^{-1}$ for the 17 $mg \cdot kg^{-1}$ dose and in the range of 0.05-200 $\mu g \cdot ml^{-1}$ for the 83 $mg \cdot kg^{-1}$ dose. Inter- and intra-day variability and the extraction recovery were determined using two quality control standards (0.3 and 9

μg·ml^{-1} for the 4 and 17 mg·kg^{-1} dose and 0.5 and 100 μg·ml^{-1} for the 83 μg·ml^{-1} dose). For zolpidem, the limit of quantification, the inter and intra-assay variability and the extraction recovery were independent of the calibration curve used and 0.05 μg·ml^{-1}, 11%, 9% and 96%, respectively.

Pharmacokinetic data analysis

Pharmacokinetic compartmental analysis was performed by fitting a standard three compartment model to the concentration-time profiles using the ADVAN11 TRANS4 subroutine within the nonlinear mixed-effect modeling software package NONMEM (NONMEM project group, University of California, San Francisco, USA). The three-compartment model was selected on the basis of visual inspection and the Akaike information criterion (Akaike, 1974). The pharmacokinetic parameters: clearance (*Cl*), the inter-compartmental clearance 2 and 3 (Q_2 and Q_3) and the volumes of distribution of compartments 1, 2 and 3 (V_1, V_2 and V_3) were estimated. The inter-individual variability of these parameters was modeled according to an exponential equation:

$$P_i = \theta_1 \cdot \exp(\eta_i), \quad (1)$$

where θ is the population estimate for parameter *P*, P_i is the individual estimate and η_i the random deviation of P_i from *P*. The values of η_i are assumed to be independently normally distributed with mean zero and variance ω^2. The residual error in the plasma drug concentration was characterized by a constant coefficient of variation (CCV) error model:

$$C_{m_{ij}} = C_{p_{ij}} \cdot (1+\varepsilon_{ij}), \quad (2)$$

where C_{pij} represents the j^{th} plasma concentration for the i^{th} individual predicted by the model. C_{mij} represents the predicted concentration, and ε_{ij} accounts for the residual deviation of the

model predicted value from the observed concentration. The value for ε was assumed to be independently normally distributed with mean zero and variance σ^2. The first order estimation method (FOCE interaction) was used to estimate the population θ, ω^2 and σ^2. Individual parameter estimates were obtained in a Bayesian posthoc step. V_{dss} and half-lives were calculated following standard procedures (Gibaldi and Perrier, 1982). Individual posthoc parameter estimates were used to calculate individual plasma concentrations at the times of the EEG measurements.

Pharmacodynamic data analysis

The concentration-effect relationships of the three doses of zolpidem were analyzed: i) using the descriptive sigmoidal E_{max} model assuming one or two sigmoidal relationships and ii) using a mechanism-based PK/PD model assuming one or two-site binding, respectively.

In the first approach the individual concentration-effect curves were fitted simultaneously to the sigmoidal E_{max} model according to:

$$E = E_0 + \frac{\alpha_1 \cdot C^{n_{H1}}}{EC_{501}^{n_{H1}} + C^{n_{H1}}} + \frac{\alpha_2 C^{n_{H2}}}{EC_{502}^{n_{H2}} + C^{n_{H2}}}, \qquad (3)$$

where E_0 is the no-drug response, α is the maximal effect the drug can produce, C is the concentration of zolpidem, EC_{50} is the concentration to produce 50% of the effect and n_H is the slope factor, which determines the steepness of the curve (i.e. the Hill-factor). The subscripts 1 and 2 refer to the parameters for the first and second sigmoidal relationship, respectively.

In the second approach, the recently proposed mechanism-based PK/PD model for $GABA_A$ receptor modulators was used (Visser *et al.*, 2002a; Visser *et al.*, 2002b; Visser *et al.*, 2003). In this model the effect is a function of the stimulus induced by the drug-receptor binding. Upon binding to the receptor, the drug produces a stimulus which is followed by a cascade of

signal-transduction processes leading to the ultimate response. A unique feature of this model is that the receptor activation process is drug-specific, whereas the stimulus-response process is system-specific. Thus, the drug-receptor activation can differ for different drugs. The stimulus-response relationship on the other hand is the same, regardless of the drug tested.

In this model, the interaction of the drug with the receptor yields a stimulus *S*, which is propagated into the ultimate effect (*E*); its relation to the stimulus is given by a function *f:*

$$E = f(S). \qquad (5)$$

In our previous analysis of the EEG effects of neuroactive steroids, benzodiazepines and other $GABA_A$ receptor modulators, the relationship *f* between the initial stimulus *(S)* and the observed EEG effect was characterized on the basis of a parabolic function (Visser *et al.*, 2002a; Visser *et al.*, 2002b; Visser *et al.*, 2003):

$$E = E_{top} - a \cdot \left(S^{d} - b\right)^2, \qquad (6)$$

where E_{top} represents the top of the parabola, *a* is a constant reflecting the slope of the parabola, $b^{1/d}$ is the stimulus for which the top of the parabola (i.e. the maximal effect, E_{top}) is reached and the exponent *d* is a parameter to account for the asymmetry of the parabola. When no drug is present the EEG effect is equal to its baseline value (E_0). Equation (6) then reduces to:

$$E_0 = E_{top} - a \cdot b^2. \qquad (7)$$

It was also shown that a variation in baseline value (E_0) is reflected in the maximal achievable response in this system (E_{top}), via parameter *a* following (Visser *et al.*, 2002b):

$$a = A \cdot E_0, \qquad (8)$$

in which A is a linear proportionality constant. Substituting equation (7) and (8) in equation (6), and rearranging yields:

$$E = E_0 \cdot \left(1 - A \cdot \left(\left(S^d\right)^2 - 2 \cdot b \cdot S^d\right)\right). \quad (9)$$

The stimulus (*S*) is a function of the concentration and contains the drug specific parameters. In the case of two-binding sites, the stimulus (*S*) is given by the formula:

$$S = \frac{e_{PD1} \cdot C}{C + K_{PD1}} + \frac{e_{PD2} \cdot C}{C + K_{PD2}}, \quad (10)$$

where K_{PD} represents *in vivo* estimated affinity and e_{PD} is the *in vivo* estimated efficacy, relative to 1, which represents maximal stimulus and biphasic EEG effects (see alphaxalone: Visser *et al.*, 2002a). The subscripts 1 and 2 refer to the parameters for the first and second binding site.

Discrimination between 1 and 2-site models

In order to determine whether the two models yield significantly different goodness-of-fits for the same data set, it is required that they are nested so that one model can be formulated as a special case of the other by setting one or more of the parameters to fixed values (Gabrielsson and Weiner, 2000). The one-site model for the sigmoidal E_{max} model (equation (3)) is a special case (a nested model) of the two-site model by fixing the parameter α_2 to 0. In a similar way, the one-site model of the mechanism-based PK/PD model is nested to the two site model by fixing the parameter e_{PD2} to 0 in equation (10). When comparing nested models, the probability that additional parameters are without effect on the sum of squares, can be estimated by an F-test (Gabrielsson and Weiner, 2000). However, in the present study maximum likelihood estimation was used for the nonlinear mixed-effect modeling and nested models were compared using the minimum value of the objective function (MVOF), which is equal to –2·log likelihood. Using likelihood ratio theory (Mood *et al.*, 1974), it can be shown that the differences between the MVOFs for two nested models follow a chi-square distribution with degrees of freedom equal to the differences in the number of parameters. Per included parameter a decrease exceeding 3.85 in

the MVOF is significant. Since the sigmoidal E_{max} model and the mechanism-based PK/PD model are not nested, it is not possible to test directly whether one of the models provides a better fit. However, Sheiner and Beal (1981) have provided some tools in comparison of structural different models by calculating the predictive performance (Yano et al., 2001). In this investigation the absolute mean prediction error (MPE) was used in order to compare the E_{max} model and the mechanism-based model following:

$$|MPE| = \left| \frac{\sum (DV_k - PRED_k)}{K} \right| \qquad (11)$$

where *DV* is the dependent variable (effect), *PRED* is the individual prediction of the *DV* and *k* is the number of observations.

In the present analysis, the parameters determining the shape of the stimulus-response relationship were fixed at values with the corresponding inter-individual variability obtained previously; *A*=9.2 (22%), *b*=0.44 (7%) and *d*=3.36 (-), respectively (Visser *et al.*, 2002b; Visser *et al.*, 2003). For the estimation of a one-site model versus the two-site model, the parameters α_2 and e_{PD2} are fixed to 0 in equation (3) and (10). Inter-individual variability for the parameters EC_{50}, and K_{PD} was modeled using an exponential error model (equation (1)) and for α, n_H, e_{PD} and E_0 using a proportional error model:

$$P_i = \theta_1 \cdot (1 + \eta_i), \qquad (12)$$

Similar to the pharmacokinetic analysis, the residual variability in the pharmacodynamics was modeled as a CCV error according to equation (2). Averaged amplitudes over 40 min of individual EEG recordings before infusion served as input for individual baseline values and it was investigated whether fixing of the baseline improved the fitting results. The FOCE interaction method was used to estimate the population θ, ω^2 and σ^2. Individual parameter

estimates were obtained in a Bayesian posthoc step. All fitting procedures were performed on an IBM-compatible personal computer (Pentium III, 450 MHz) running under Windows NT 4.0 and Visual-NM 2.2.2. (RDPP, Montpellier, France) with the use of the Microsoft FORTRAN PowerStation 4.0 compiler with NONMEM version V.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparison test. In case of non-homogeneity, as determined by Bartlett's test, the non-parametric Kruskall-Wallis test was used. Statistical tests were performed using InStat version 3.0 for Windows (GraphPad, San Diego, USA). All data are represented as mean ± S.E.M and the significance level was set to $P < 0.05$.

RESULTS

Pharmacokinetic analysis

The concentration-time profiles of the three dosages of zolpidem were best described using a three-compartment pharmacokinetic model. The observed and predicted pharmacokinetic profiles are depicted in figure 1. The population pharmacokinetic parameter estimates for the simultaneous analysis and the corresponding inter- and intra-individual variability are summarized in table 1. The determination of zolpidem protein-binding has been described previously (Visser *et al.*, 2003). The free fraction in plasma was 4.2 ± 0.1% and independent of the concentration.

Pharmacodynamic analysis

Figure 2 shows the observed and predicted zolpidem EEG effect (amplitude in 11.5-30 Hz band) *versus* time profiles per dosing group. The vehicle did not have influence on the EEG effect compared to baseline. It was observed that the maximal EEG effect increased with increasing dose and that the concentration-effect relationship is shifted rightwards with increasing dose. Due to the long pharmacokinetic elimination phase, the effects of the 83 $mg \cdot kg^{-1}$ dose do not fully return to baseline in the time-course of the experiment.

The zolpidem plasma concentrations were calculated at the time points of effect measurements using the individual posthoc pharmacokinetic parameter estimates. The resulting concentration-effect relationships were fitted to two models: 1) nested models based on the sigmoidal E_{max} equation with one and two sigmoidal relationships and the 2) nested models based on the previously postulated mechanism-based PK/PD model with one or two-site binding.

In the analysis according to the sigmoidal E_{max} model, the use of two sigmoidal relationships *versus* a one sigmoidal relationship resulted in a significant reduction in the

objective function of 164 ($p < 0.05$). However, in the fitting procedures, difficulties were observed with respect to parameter variability of the two slope factors, therefore the error models on the slope factors were fixed at 0. Parameter estimates for $EC_{50\text{-}1}$ and $EC_{50\text{-}2}$ were 1990 ± 725 and 28,800 ± 11,500 ng·ml^{-1} and for the E_{max1} and E_{max2} 4.5 ± 0.7 µV and 11.4 ± 1.5 µV, respectively. The population parameter estimates are summarized in table 2.

The use of a two-site binding model *versus* a one-site binding model within the mechanism-based PK/PD model resulted in a significant reduction in the objective function of 438 ($p < 0.05$). The model was able to successfully describe all individual concentration-effect relationships. The pharmacodynamic parameter estimates are summarized in table 3. Parameter estimates for K_{PD1} and K_{PD2} were 97 ± 40 and 33,100 ± 14,800 ng·ml^{-1}, whereas for e_{PD1} and e_{PD2} 0.47 ± 0.02 and 0.34 ± 0.05 were estimated, respectively.

In comparision of the two-site Emax model and the two-site mechansim-based model the absolute mean prediction errors were calculated and were 0.824 ± 0.693 and 0.779 ± 0.661, respectively. This was not significantly different. Visual inspection revealed that the data were best described by the population fit of the mechanism-based model. The population prediction and individual predictions of the mechanism-based two-site model for all individual rats are shown in figure 3. It is important that in a number of animals, especially from the highest dose groups, a tendency towards a biphasic concentration-effect relationship was observed, which was successfully described with the mechanism-based model, while the descriptive two-site model failed to describe this observed biphasic pattern. This is explained in figure 4. The population drug-receptor interaction is shown in panel A, whereas the predicted stimulus-response relationship for each individual is shown in panel B. The observed stimulus-response relationship for zolpidem was consistent with the thick black line which represent the stimulus-response relationship as was previously found for neuroactive steroids. Furthermore, the stimulus-response

relationship was not different between the dosages of zolpidem. For some individuals, the stimulus-response relationship reached the top of the parabola, which explains why a tendency towards a biphasic concentration-effect was observed.

DISCUSSION

In the present investigation the concentration-effect relationship of zolpidem, a well known subtype selective ligand for the $GABA_A$ receptor, was studied in conscious rats using the change in β-frequency range of the EEG as pharmacodynamic endpoint. The β-frequency range is known to reflect $GABA_A$ receptor activation (Mandema and Danhof, 1992). However, it was not known whether $GABA_A$ receptor subtype selectivity could be quantitatively determined in the EEG. Qualitatively, it has been found in the human EEG that zolpidem induced a higher maximum increase within the 20-30 Hz frequency band compared to benzodiazepines (Depoortere *et al.*, 1988; Patat *et al.*, 1994). Quantitatively, the EEG effects of zolpidem were reported to be higher than midazolam and bretazenil in rats (Tuk *et al.*, 2002).

The present results suggest that the activation of two receptor subtypes by zolpidem can be characterized and quantified in the β-frequency range of the EEG using an integrated mechanism-based PK/PD approach. In contrast, the descriptive sigmoidal E_{max} model did not adequately describe and explain the observations although the absolute mean prediction error was not significantly different. In comparison to traditional descriptive PK/PD models such as the sigmoidal E_{max} model, mechanism-based PK/PD models are considered of interest, because of their much improved properties for extrapolation and prediction. An essential feature of mechanism-based PK/PD models is the separation of the drug-specific properties from the system specific properties, which enables *in vitro-in vivo* prediction and the interspecies extrapolation and prediction (Van der Graaf and Danhof, 1997). Recently, a full parametric mechanism-based model has been developed and successfully applied to a wide array of $GABA_A$ receptor modulators, including (synthetic) neuroactive steroids, benzodiazepines and other ligands for the benzodiazepine site and inverse agonists (Visser *et al.*, 2002a; Visser *et al.*, 2002b; Visser *et al.*, 2003). This model features a monophasic receptor activation model in combination with a

biphasic transducer model. The receptor activation process is described by a hyperbolic function, whereas a parabolic function is used for the description of the transducer function (see figure 4). For a wide array of $GABA_A$ receptor modulators it has been shown that on the basis of this model estimates of *in vivo* receptor affinity and intrinsic efficacy can be obtained, which were closely correlated with estimates obtained in *in vitro* bio-assays (Visser *et al.*, 2003).

In the present investigation it was shown that the mechanism-based PK/PD modeling approach yielded affinity estimates for two binding sites (97 ± 40 and 33,100 ± 14,800 $ng \cdot ml^{-1}$, corresponding to 315 nM and 107 µM) with a ratio between the K_{PD1} and K_{PD2} of 340 fold. It is not known whether free concentrations or total concentrations are the major determinant in the generation of the zolpidem effect. Unbound K_{PD1} and K_{PD2} estimates were 12.6 nM and 4.2 µM respectively. *In vitro*, zolpidem was found to discriminate between two flumazenil binding sites in neonatal brain with an IC_{50} value ratio of more than 200 fold (300 nM and 40 µM), whereas in adult rat brain sometimes three binding sites have been found with K_d values of 10-20 nM, 200-300 nM and 4-10 µM (Benavides *et al.*, 1993; Ruano *et al.*, 1992). The unbound values, corresponding to the high and low affinity site in adult brain might indicate that protein binding is a determinant for the effect of zolpidem, however, this remains to be investigated.

The relative intrinsic efficacies that were estimated were 0.47 ± 0.02 and 0.34 ± 0.05, suggesting that zolpidem can exert its effect via two receptor subtypes at high doses, despite the low affinity for one subtype. It can be speculated that the changes in the β-frequency of the EEG reflects the summation of effects of all activated $GABA_A$ receptor subtypes. Since benzodiazepines have equal affinity for the $GABA_A$ receptor subtypes consisting of combinations with various α-subunits (Pritchett *et al.*, 1989), it cannot be distinguished *in vivo* whether the $GABA_A$ receptor-mediated EEG effects of benzodiazepines are due to the activation of one or

several receptor subtypes. However, due to large differences in affinity, a heterogeneous receptor activation by zolpidem could be distinguished *in vivo*.

The dose-dependent EEG effects found in this investigation are not likely due to differences in the pharmacokinetics. In PK/PD modeling, the time course of the concentration is linked to the time course of the effect for each individual, thereby taking these differences into account. Furthermore, the pharmacokinetic differences are probably due to the very slow elimination phase which were below detection limit for the lowest dose. In addition, protein binding of zolpidem was independent of the concentration added (Visser *et al.*, 2003).

Interestingly, for some individuals receiving the highest dose, the EEG effect resulted in a tendency towards a biphasic pattern (see figure 3). This is consistent with the expectation of the mechanism-based model (see figure 4). Although the biphasic stimulus-response relationship was proposed based on the biphasic concentration-effect relationships of neuroactive steroids, it is shown in this investigation that this biphasic stimulus-response relationship is indeed a system-related process. The maximal EEG effect (E_{top}) can be predicted from the baseline values (see equations (7) and (8) and its value is estimated between 25 and 30 µV. The individual predictions of E_{top} are between 25 and 30 µV and it can be derived from figure 3 that zolpidem at the highest dose activated the system to E_{top} values. Recently, the pharmacodynamic interaction of zolpidem with ethanol was analyzed using a mechanism-based model where no *a priori* assumptions were made of the shape of the stimulus-response relationship in contrast to our parameterized biphasic stimulus-response function (Tuk *et al.*, 2002). In that investigation a remarkably similar stimulus-response relationship was found for zolpidem, which was not altered in the presence of ethanol, indicating that the pharmacodynamic interaction between zolpidem and ethanol occurs at receptor level (Tuk *et al.*, 2002). This underscores that a mechanism-based PK/PD approach constitutes a

realistic approach to the characterization of the effects of $GABA_A$ receptor modulators *in vivo* regarding the $GABA_A$ receptor mediated EEG effects.

In conclusion, the mechanism-based model describef and explained the dose-dependent EEG effects of zolpidem, in contrast to the descriptive sigmoidal E_{max} model. This mechanism-based PK/PD model suggests that activation of two receptor subtypes by zolpidem can be characterized and quantified *in vivo* using EEG as pharmacodynamic endpoint.

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Table 1. Population pharmacokinetic parameter estimates for *Cl*, Q_2, Q_3, V_1, V_2 and V_3 ($\theta \pm$ S.E.) with the corresponding inter-individual coefficient of variation (CV%) and 95% confidence interval (C.I.) for all doses of zolpidem modeled simultaneously. Intra-individual residual variation was 21%.

Group	*Cl*	Q_2	Q_3	V_1	V_2	V_3
	$(ml \cdot min^{-1} \cdot kg^{-1})$	$(ml \cdot min^{-1} \cdot kg^{-1})$	$(ml \cdot min^{-1} \cdot kg^{-1})$	$(l \cdot kg^{-1})$	$(l \cdot kg^{-1})$	$(l \cdot kg^{-1})$
Total (n=24)	31.8 ± 3.6	67.7 ± 9.9	5.4 ± 1.2	0.25 ± 0.05	0.43 ± 0.04	1.53 ± 0.40
CV%	(53%)	(<1%)	(62%)	(75%)	(29%)	(33%)
95 % C.I.	25 - 40	47 - 88	3 - 8	0.15 - 0.34	0.36 - 0.50	0.74 - 2.32

Table 2. Population pharmacodynamic parameter estimates for the descriptive pharmacodynamic model: E_{max1}, $EC_{50\text{-}1}$, n_{H1}, E_{max2}, $EC_{50\text{-}2}$, n_{H2} with the corresponding inter-individual coefficient of variation (CV%) for all doses of zolpidem modeled simultaneously assuming two-site binding. Intra-individual residual variation was 9%.

Group	E_0 (μV)	E_{max1} (μV)	$EC_{50\text{-}1}$ ($ng \cdot ml^{-1}$)	n_{H1}	E_{max2} (μV)	$EC_{50\text{-}2}$ ($ng \cdot ml^{-1}$)	n_{H2}
Total (n=24)	11.8 ± 0.4	4.5 ± 0.7	1988 ± 725	3.5 ± 0.65	11.4 ± 1.5	28,823 ± 11,478	0.63 ± 0.2
CV%	(17%)	(74%)	(123%)	(-)	(166%)	(178%)	(-)

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Table 3. Population pharmacodynamic parameter estimates for the mechanism-based pharmacodynamic model: $E_{0,}$ e_{PD1}, K_{PD1}, e_{PD2} and K_{PD2} with the corresponding inter-individual coefficient of variation (CV%) for all doses of zolpidem modeled simultaneously for two-site binding. Intra-individual residual variation was 9%.

Group	E_0	e_{PD1}	K_{PD1}	e_{PD2}	K_{PD2}
	(μV)		*(ng·ml^{-1})*		*(ng·ml^{-1})*
Population (n=24)	11.8 ± 0.4	0.47 ± 0.02	97 ± 40	0.34 ± 0.05	33,100 ± 14,800
CV%	(16%)	(19%)	(177%)	(-)	(175%)

Legends to the figures:

Figure 1. Pharmacokinetics of zolpidem. Observed (open circles), individual predicted (thin lines) and population predicted (thick lines) concentration-time profiles upon administration of 4 $mg \cdot kg^{-1}$, 17 $mg \cdot kg^{-1}$ and 83 $mg \cdot kg^{-1}$ zolpidem in 5 min. Time is depicted on the x-axis and the concentration is depicted on the y-axis on a logarithmic scale. Infusions started at t=0 min

Figure 2: Pharmacodynamics of zolpidem. observed (open circles) and predicted (thin lines) EEG effect *versus* time profiles upon i.v. administration of the vehicle, 4 $mg \cdot kg^{-1}$, 17 $mg \cdot kg^{-1}$ and 83 $mg \cdot kg^{-1}$ zolpidem in 5 min. Time is depicted on the x-axis and the effect is depicted on the y-axis as amplitude in β-frequency range. Predictions represent the best fitsusing the two-site mechanism-based PK/PD model. Infusions started at t=30 min except for the 4 $mg \cdot kg^{-1}$ dose which started on t=45 min.

Figure 3: Observed and predicted individual concentration-effect profiles. Open circles are the the observed amplitudes, the black line is the individual prediction and the gray line is the population prediction. Predictions represent the best fitsusing the two-site mechanism-based PK/PD model. The dose and rat number is depicted in the graphs. Concentration in $ng.ml^{-1}$ is depicted on the x-axis and the effect is depicted on the y-axis as amplitude in β-frequency range.

Figure 4: **Panel A:** *Drug-receptor interaction*. The relationship between the plasma concentration of zolpidem was simulated on the basis of the population parameter estimates for e_{PD1}, e_{PD2}, K_{PD1} and K_{PD2}, which are depicted as dots with the standard error of the prediction. Plasma concentration ($ng \cdot ml^{-1}$) is depicted on the x-axis on a logarithmic scale and the stimulus is

depicted on the y-axis. **Panel B:** *Stimulus-effect relationship.* The stimulus effect relationship as described by the parabolic function for zolpidem. Dots represent the observed amplitudes for zolpidem. The lines represent the best fitted stimulus effect relationship for each individual and the thick line represent the stimulus-response relationship as defined for the neuroactive steroids.

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Figure 1:

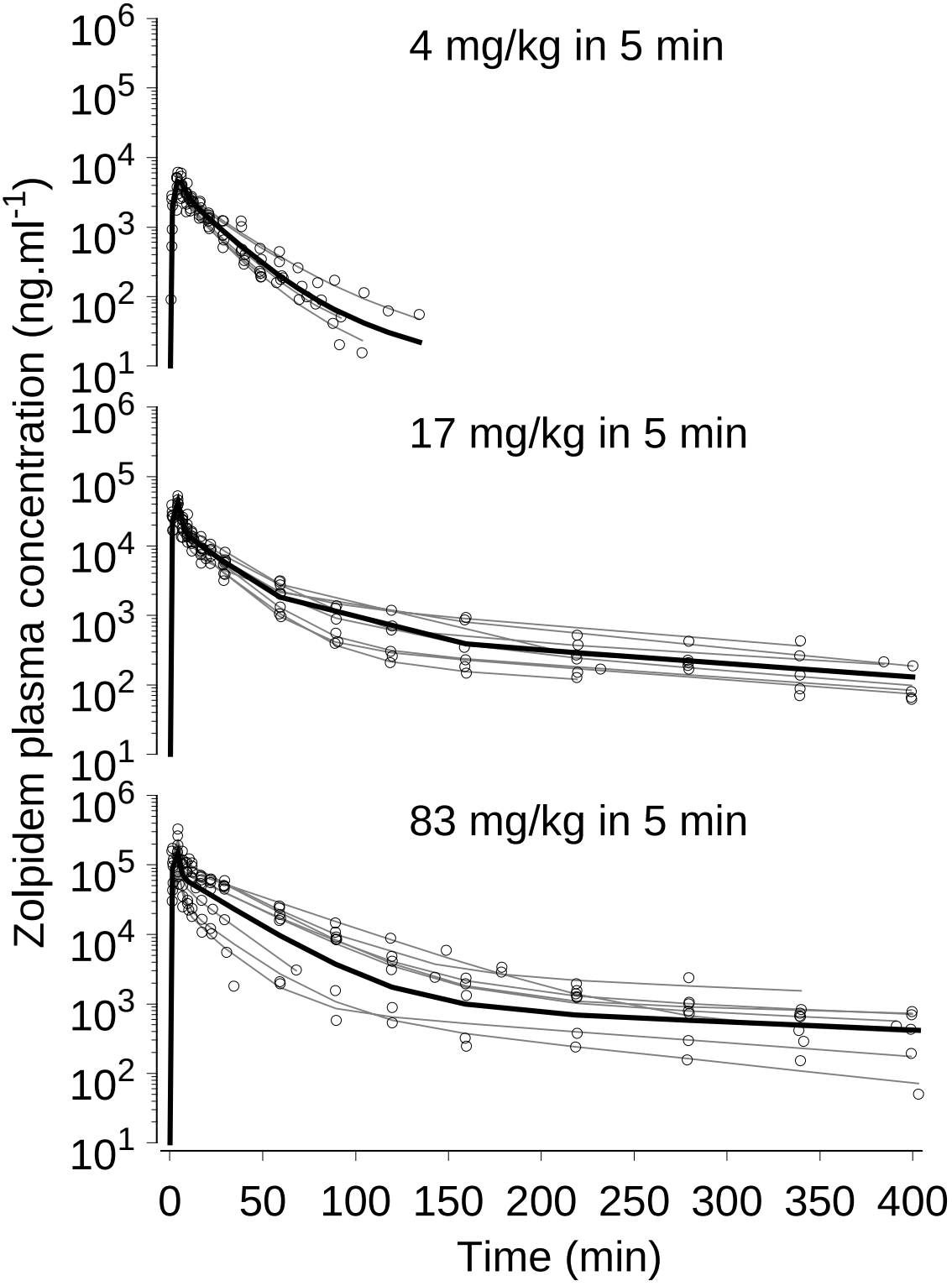

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Figure 2:

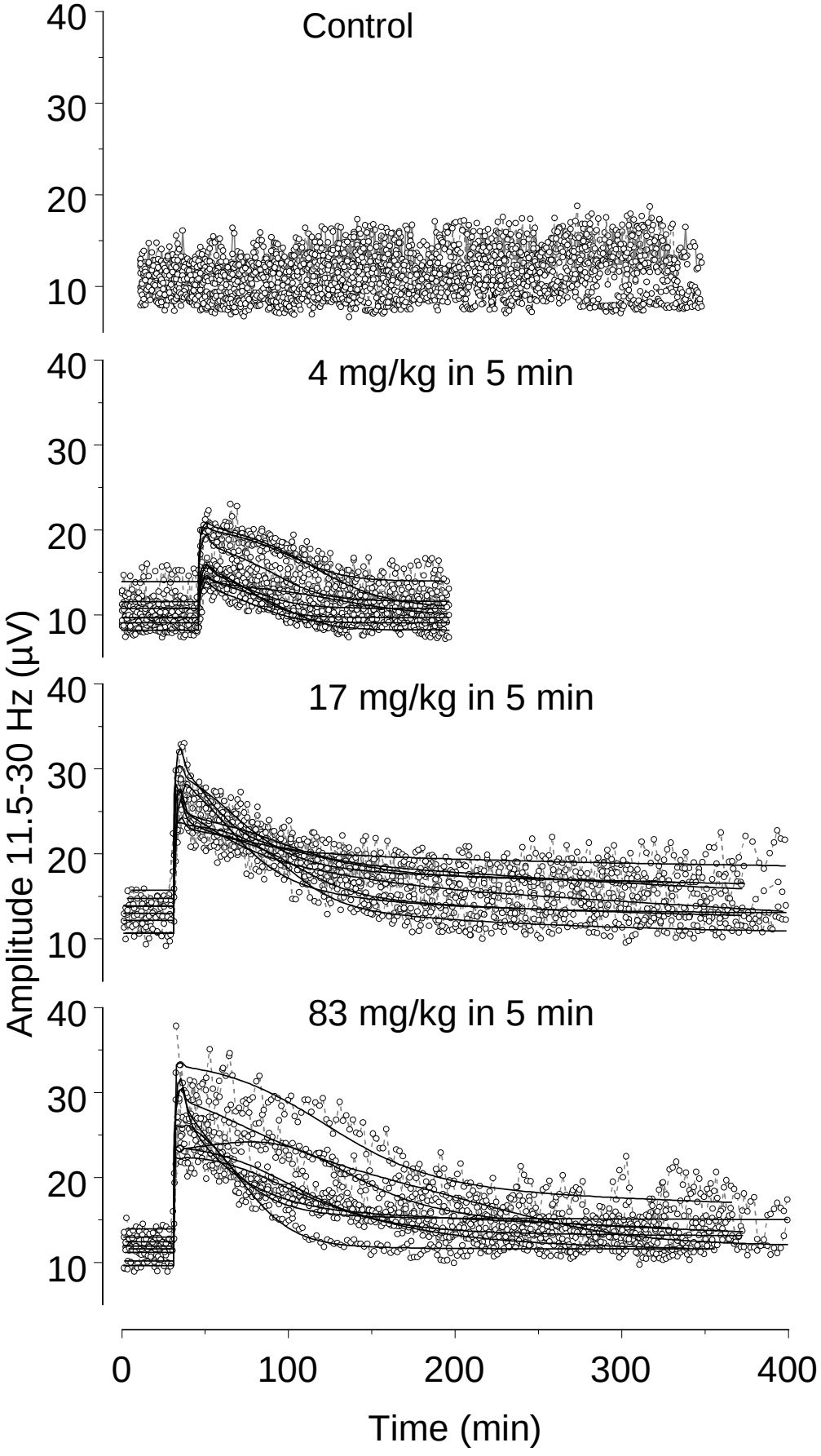

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Figure 3

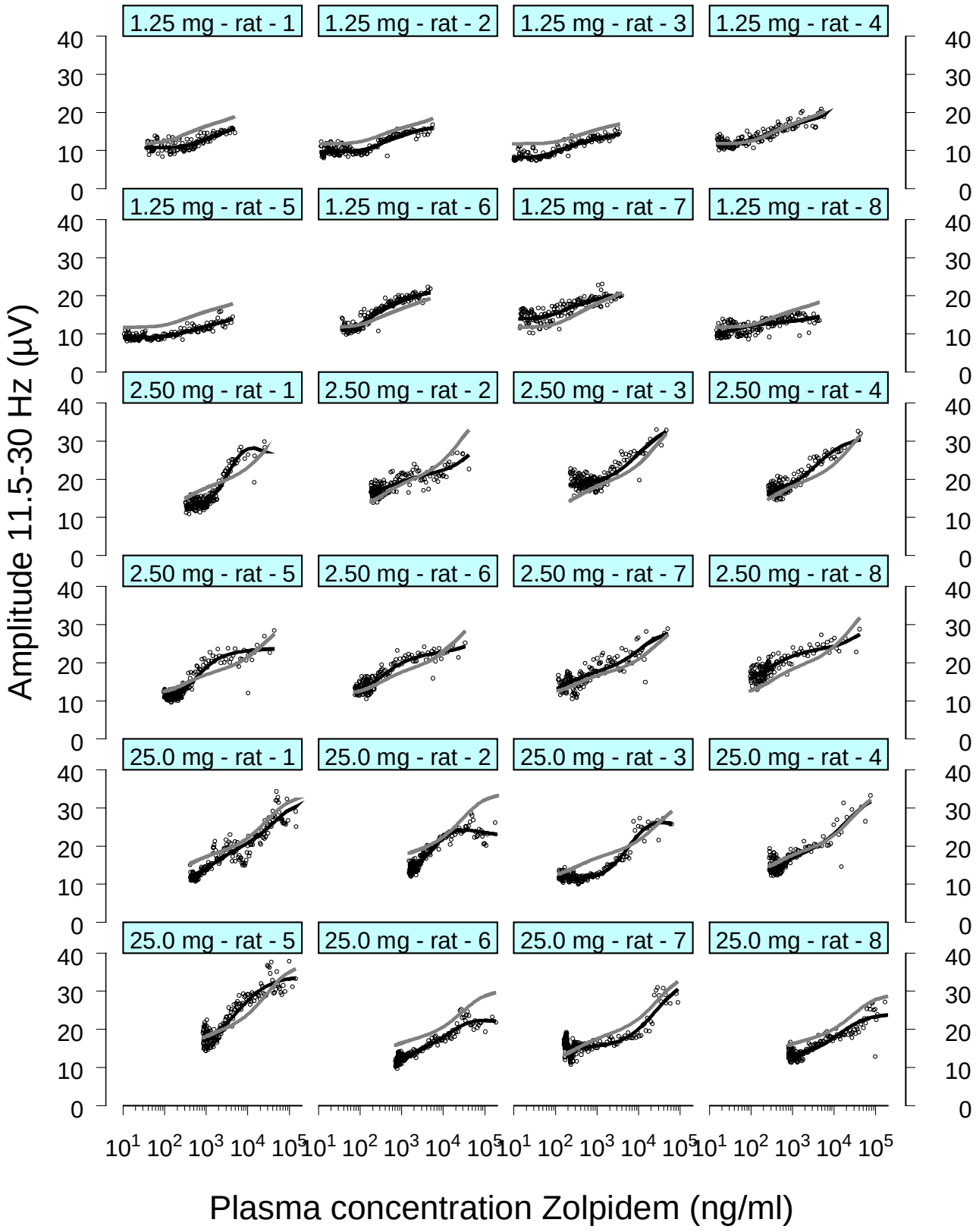

Figure 4.