

Dose-Dependent EEG Effects of Zolpidem Provide Evidence for GABA_A Receptor Subtype Selectivity in Vivo

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Received October 2, 2002; accepted November 22, 2002

ABSTRACT

Zolpidem is a nonbenzodiazepine GABA_A receptor modulator that 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 1) the descriptive model and 2) a novel mechanism-based pharmacokinetic/pharmacodynamic (PK/PD) model for GABA_A receptor modulators that aims to separate 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 with 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 $33,100 \pm 14,800$ ng · 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 (for review, see 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 (BZ₁) and ω_2 (BZ₂), which were classified on the basis of differing affinities of CL 218,872 and zolpidem, respectively. In the meantime, it has been shown in in vitro investigations that zolpidem, which is a hypnotic of the imidazopyridine class, differs from conventional benzodiazepines (e.g., flunitrazepam and diazepam) and other hypnotics (zopiclone). Zolpi-

dem 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 μ M), whereas benzodiazepines have equal affinity for the various GABA_A receptor subtypes (Pritchett et al., 1989; Ruano et al., 1992; Benavides et al., 1993; Luddens and Korpi, 1995). 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 (Perrault et al., 1988; Barnard et al., 1998).

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

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Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.102.044859.

GABA_A receptors in the transduction of the discriminatory stimulus effects of zolpidem at higher doses, compared with the classical benzodiazepine diazepam *in vivo*. Furthermore, it has been demonstrated in mice that the sedative-hypnotic and anticonvulsant 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 an array of GABA_A receptor modulators, including neuroactive steroids, benzodiazepines, imidazopyridines, cyclopyrrolones, and β -carbolines (Visser et al., 2002a,b, 2003). This model comprises a separate characterization of 1) the receptor activation process and 2) the signal transduction process. In this model, the receptor activation process is described by a hyperbolic function, whereas 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 that are closely correlated to estimates obtained in *in vitro* bioassays, 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.

Materials and 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 ± S.D.); Broekman Breeding Facilities, Someren, The Netherlands] were used. After surgery, the rats were housed individually in standard plastic cages with a normal 12-h day/night schedule (lights on 7:00 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 (F₁ and F_r), 3 mm anterior and 3.5 mm lateral (C₁ and C_r), and 3 mm posterior and 2.5 mm lateral (O₁ and O_r) to lambda, where a reference electrode was placed (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% poly-vinylpyrrolidone 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 · kg⁻¹ i.m. medetomidine hydrochloride (Domitor; Pfizer, Capelle a/d IJssel, The Netherlands) and 1 mg · kg⁻¹ s.c. ketamine base (Ketalar; Parke-Davis, Hoofddorp, The Netherlands). After the first surgery, 4 mg of ampicillin (A.U.V., Cuijk, The Netherlands) was administered to aid recovery.

Treatment and Dosages. Zolpidem was obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Infusion solutions of zolpidem were prepared in 250 μ l of 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 · 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 (maximum 450 min). Bipolar EEG leads on the left hemisphere (F₁-C₁) 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 1401_{plus} interface (CED, Cambridge, UK). The signal was fed into an 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., Indianapolis, IN). 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). 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.

Serial arterial blood samples were taken at predefined 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 rpm 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 of 0.1 M NaOH, 50 μ l of clobazam as internal standard (1 μ g/ml in MeOH) was added, and the mixture was extracted with 5 ml of dichloromethane/petroleum ether (45:55, v/v). The mixture was vortexed for 5 min and subsequently centrifuged for 15 min at 5000 rpm. The samples were placed at -20°C to freeze the water phase. The organic phase was transferred to a clean tube and evaporated under reduced pressure at 37°C. The residue was dissolved in 150 μ l of mobile phase of which 40 μ l 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 from Waters, Milford, MA), a 150 × 4.6 mm C18 5- μ m column (Alltech BV, Breda, The Netherlands) equipped with a hand-packed C18 guard column (20 mm × 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 C-R3A integrator (Shimadzu, 's Hertogenbosch, The Netherlands). Linear calibration curves were obtained in the range of 0.05 to 10 μ g · ml⁻¹ for the 4 mg · kg⁻¹ dose, in the range of 0.05 to 50 μ g · ml⁻¹ for the 17 mg · kg⁻¹ dose and in the range of 0.05 to 200 μ g · ml⁻¹ for the 83 mg · kg⁻¹ dose. Inter- and intraday variability and the extraction recovery were determined using two quality control standards (0.3 and 9 μ g · ml⁻¹ for the 4 and 17 mg · kg⁻¹ dose and 0.5 and 100 μ g · ml⁻¹ for the 83 mg · kg⁻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 μ g · 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, San Francisco, CA). The three-compartment model was selected on the basis of visual inspection and the Akaike information criterion (Akaike, 1974). The pharmacokinetic parameters clearance (CL); intercompartmental clearances 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 interindividual 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 error model:

$$C_{mij} = C_{pij} \cdot (1 + \epsilon_{ij}) \quad (2)$$

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 was used to estimate the population θ , ω^2 , and σ^2 . Individual parameter estimates were obtained in a Bayesian post hoc step. V_{dss} and half-lives were calculated following standard procedures (Gibaldi and Perrier, 1982). Individual post hoc 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 by 1) using the descriptive sigmoidal E_{max} model assuming one or two sigmoidal relationships; and 2) 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 the following equation:

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

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,b, 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 that 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 :

$$E = f(S) \quad (4)$$

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,b, 2003):

$$E = E_{top} - a \cdot (S^d - b)^2 \quad (5)$$

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 5 then reduces to the following:

$$E_0 = E_{top} - a \cdot b^2 \quad (6)$$

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

$$a = A \cdot E_0 \quad (7)$$

in which A is a linear proportionality constant. Substituting eqs. 6 and 7 in eq. 5, and rearranging yields the following:

$$E = E_0 \cdot (1 - A \cdot ((S^d)^2 - 2 \cdot b \cdot S^d)) \quad (8)$$

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

$$S = \frac{e_{PD1} \cdot C}{C + K_{PD1}} + \frac{e_{PD2} \cdot C}{C + K_{PD2}} \quad (9)$$

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 alphasalone; Visser et al., 2002a). The subscripts 1 and 2 refer to the parameters for the first and second binding site, respectively.

Discrimination between One- and Two-Site Models. 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 (eq. 3) is a special case (a nested model) of the two-site model by fixing the parameter α_2 to zero. 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 zero in eq. 9. 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 $-2 \cdot \log$ 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. Because 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 was used to compare the E_{\max} model and the mechanism-based model following

$$|\text{MPE}| = \left| \frac{\sum (\text{DV}_k - \text{PRED}_k)}{K} \right| \quad (10)$$

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 interindividual variability obtained previously; $A = 9.2$ (22%), $b = 0.44$ (7%), and $d = 3.36$ (-), respectively (Visser et al., 2002b, 2003). For the estimation of a one-site model versus the two-site model, the parameters α_2 and $e_{\text{PD}2}$ are fixed to zero in eqs. 3 and 9. Interindividual variability for the parameters EC_{50} , and K_{PD} was modeled using an exponential error model (eq. 1) and for α , n_{H} , e_{PD} , and E_0 using a proportional error model:

$$P_i = \theta_1 \cdot (1 + \eta_i) \quad (11)$$

Similar to the pharmacokinetic analysis, the residual variability in the pharmacodynamics was modeled as a constant coefficient of variation error according to eq. 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 first-order estimation method was used to estimate the population θ , ω^2 , and σ^2 . Individual parameter estimates were obtained in a Bayesian post hoc 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 and a Tukey-Kramer multiple comparison test. In case of nonhomogeneity, as determined by Bartlett's test, the nonparametric Kruskal-Wallis test was used. Statistical tests were performed using InStat version 3.0 for Windows (Graph-Pad Software Inc., San Diego, CA). All data are represented as mean \pm 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 Fig. 1. The population pharmacokinetic parameter estimates for the simultaneous analysis and the corresponding inter- and intraindividual 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 $4.2 \pm 0.1\%$ 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 with 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 $83 \text{ mg} \cdot \text{kg}^{-1}$ 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 post

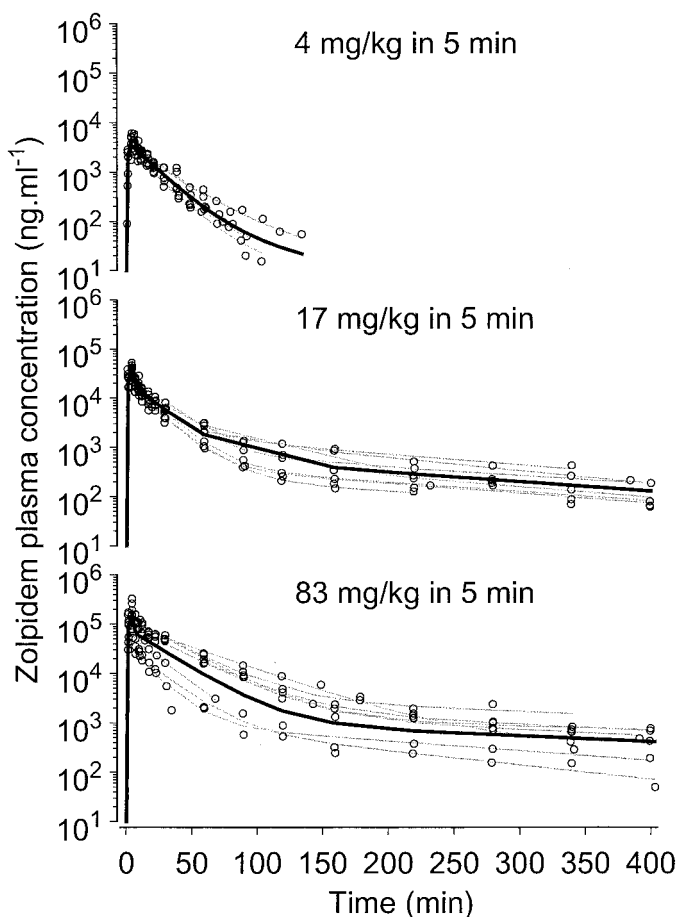


Fig. 1. Pharmacokinetics of zolpidem. Observed (open circles), individual predicted (thin lines), and population predicted (thick lines) concentration-time profiles upon administration of $4 \text{ mg} \cdot \text{kg}^{-1}$, $17 \text{ mg} \cdot \text{kg}^{-1}$, and $83 \text{ mg} \cdot \text{kg}^{-1}$ 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.

hoc 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 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 zero. Parameter estimates for EC_{50-1} and EC_{50-2} were $1,990 \pm 725$ and $28,800 \pm 11,500 \text{ ng} \cdot \text{ml}^{-1}$ and for the $E_{\max 1}$ and $E_{\max 2}$ 4.5 ± 0.7 and $11.4 \pm 1.5 \mu\text{V}$, 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 $K_{\text{PD}1}$ and $K_{\text{PD}2}$ were 97 ± 40 and

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 interindividual coefficient of variation (CV%) and 95% confidence interval (CI) for all doses of zolpidem modeled simultaneously. Intraindividual residual variation was 21%.

Group	CL	Q_2	Q_3	V_1	V_2	V_3
		$ml \cdot min^{-1} \cdot kg^{-1}$			$l \cdot kg^{-1}$	
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% CI	25–40	47–88	3–8	0.15–0.34	0.36–0.50	0.74–2.32

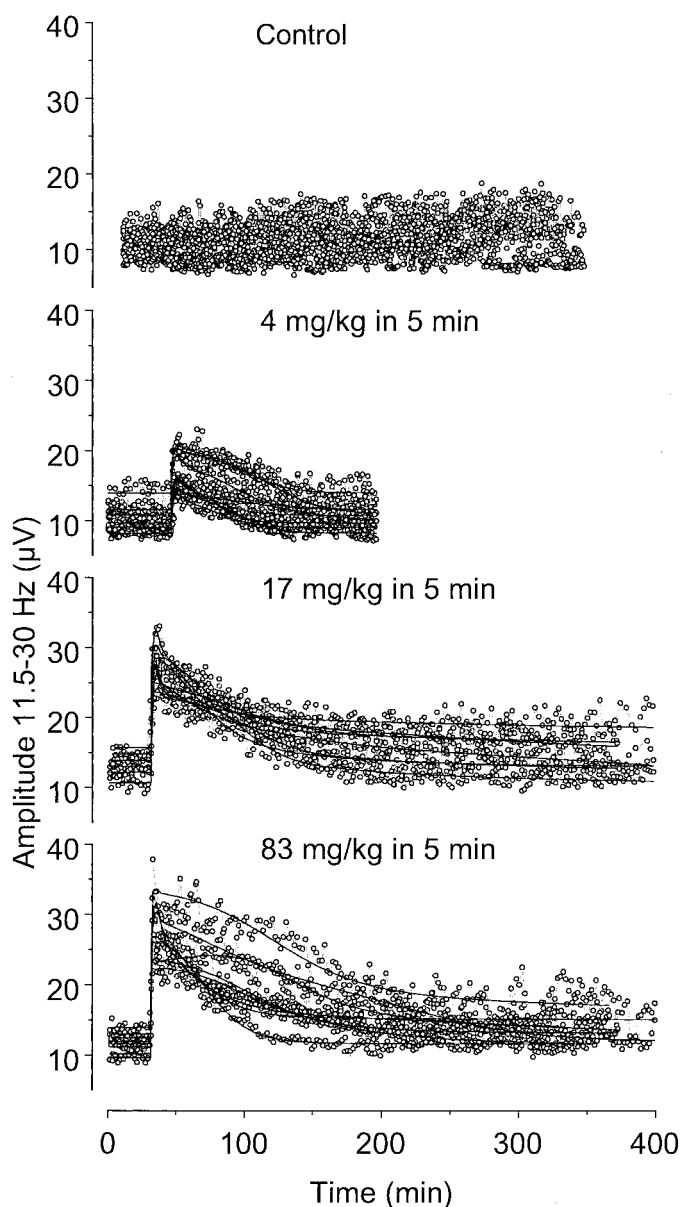


Fig. 2. Pharmacodynamics of zolpidem. Observed (open circles) and predicted (thin lines) EEG effect versus time profiles upon i.v. administration of the vehicle, $4 \text{ mg} \cdot \text{kg}^{-1}$, $17 \text{ mg} \cdot \text{kg}^{-1}$, and $83 \text{ mg} \cdot \text{kg}^{-1}$ zolpidem in 5 min. Time 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 fits using the two-site mechanism-based PK/PD model. Infusions started at $t = 30$ min except for the $4 \text{ mg} \cdot \text{kg}^{-1}$ dose, which started on $t = 45$ min.

$33,100 \pm 14,800 \text{ ng} \cdot \text{ml}^{-1}$, whereas for e_{PD1} and e_{PD2} 0.47 ± 0.02 and 0.34 ± 0.05 were estimated, respectively.

In comparison with the two-site E_{max} model and the two-site mechanism-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 Fig. 3. It is important that in a number of animals, especially from the highest dose groups, a tendency toward a biphasic concentration-effect relationship was observed, which was successfully described with the mechanism-based model, whereas the descriptive two-site model failed to describe this observed biphasic pattern. This is explained in Fig. 4. The population drug-receptor interaction is shown in A, whereas the predicted stimulus-response relationship for each individual is shown in Fig. 4B. The observed stimulus-response relationship for zolpidem was consistent with the thick black line that 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 toward 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- to 30-Hz frequency band compared with benzodiazepines (Depoortere et al., 1988; Patat et al., 1994). Quantitatively, the EEG effects of zolpidem were reported to be higher than those of 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

TABLE 2

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

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

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 interindividual coefficient of variation (CV%) for all doses of zolpidem modeled simultaneously for two-site binding.

Intraindividual residual variation was 9%.

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

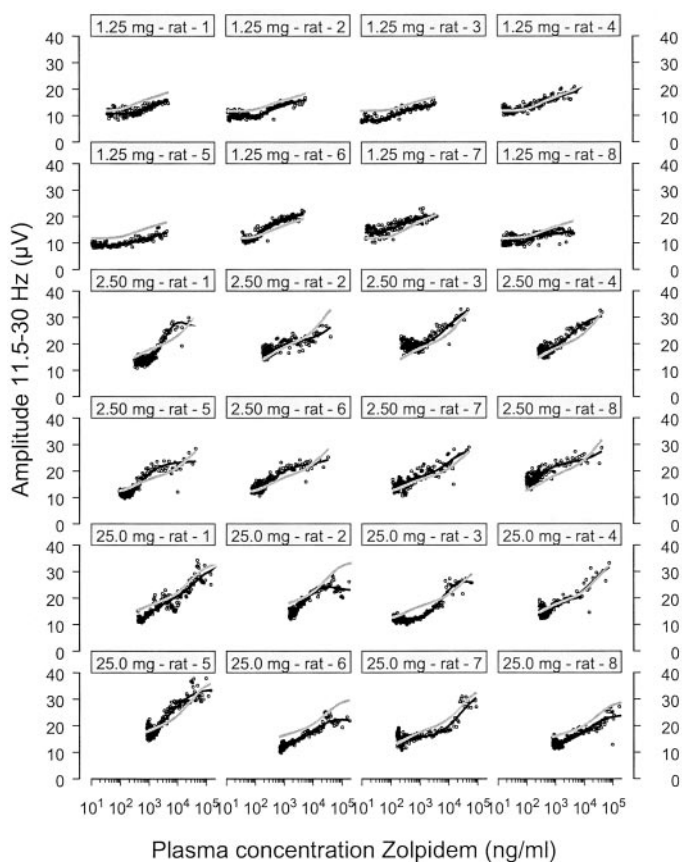


Fig. 3. Observed and predicted individual concentration-effect profiles. Open circles are the observed amplitudes, the black line is the individual prediction, and the gray line is the population prediction. Predictions represent the best fits using the two-site mechanism-based PK/PD model. The dose and rat number is depicted in the graphs. Concentration in nanograms per milliliter is depicted on the x-axis and the effect is depicted on the y-axis as amplitude in β -frequency range.

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

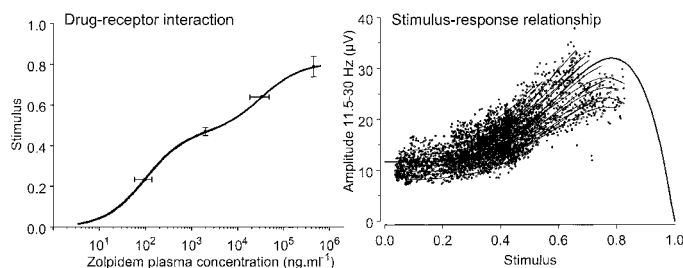


Fig. 4. A, drug-receptor interaction. The relationship between the plasma concentration of zolpidem was simulated on the basis of the population parameter estimates for e_{PD1} , e_{PD2} , K_{PD1} , and K_{PD2} , which are depicted as dots with the standard error of the prediction. Plasma concentration (nanograms per milliliter) is depicted on the x-axis on a logarithmic scale and the stimulus is depicted on the y-axis. 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.

benzodiazepine site and inverse agonists (Visser et al., 2002a,b, 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 (Fig. 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 that are closely correlated with estimates obtained in in vitro bioassays (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 $33,100 \pm 14,800$ ng \cdot ml⁻¹, corresponding to 315 nM and 107 μ M) with a ratio between the K_{PD1} and K_{PD2} of 340-fold. It is not known whether free concentration or total concentration is the major determinant in the generation of the zolpidem effect. Unbound K_{PD1} and K_{PD2} estimates were 12.6 nM and 4.2 μ M, respectively. In vitro, zolpidem was found to discriminate between two flumazenil binding sites in neonatal brain with an IC₅₀ value ratio of more than 200-fold (300 nM and 40 μ M), whereas in adult rat brain sometimes three binding sites have been found with K_d values of 10 to 20 nM, 200 to

300 nM, and 4 to 10 μ M (Ruano et al., 1992; Benavides et al., 1993). 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 reflect the summation of effects of all activated GABA_A receptor subtypes. Because 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 toward a biphasic pattern (Fig. 3). This is consistent with the expectation of the mechanism-based model (Fig. 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 E_{top} can be predicted from the baseline values (see eqs. 6 and 7) and its value is estimated between 25 and 30 μ V. The individual predictions of E_{top} are between 25 and 30 μ V, and it can be derived from Fig. 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 described 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.

Acknowledgments

We gratefully acknowledge Erica Tukker and Josy Gubbens-Stibbe for technical assistance.

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