Mechanism-Based Pharmacokinetic/Pharmacodynamic Modeling of the Electroencephalogram Effects of GABA$_A$ Receptor Modulators: In Vitro-in Vivo Correlations

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ABSTRACT

A mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) model for neuroactive steroids, comprising a separate characterization of 1) the receptor activation process and 2) the stimulus-response relationship, was applied to various nonsteroidal GABA$_A$ receptor modulators. The EEG effects of nine prototypical GABA$_A$ receptor modulators (six benzodiazepines, one imidazopyridine, one cyclopyrrolone, and one β-carboline) were determined in rats in conjunction with plasma concentrations. Population PK/PD modeling revealed monophasic concentration-EEG effect relationships with large differences in potency (EC$_{50}$) and intrinsic activity between the compounds. The data were analyzed on the basis of the mechanism-based PK/PD model for (synthetic) neuroactive steroids on the assumption of a single and unique stimulus-response relationship. The model converged yielding estimates of both the apparent in vivo receptor affinity (K_{PD}) and the in vivo intrinsic efficacy (e_{PD}). The values of K_{PD} ranged from 0.41 ± 0 ng·ml^{-1} for bretazenil to 436 ± 72 ng·ml^{-1} for clobazam and the values for e_{PD} from −0.27 ± 0 for methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate to 0.54 ± 0.02 for diazepam. Significant linear correlations were observed between K_{PD} for unbound concentrations and the affinity in an in vitro receptor bioassay ($r = 0.93$) and between e_{PD} and the GABA-shift in vitro ($r = 0.95$). The findings of this investigation show that the in vivo effects of nonsteroidal GABA$_A$ receptor modulators and (synthetic) neuroactive steroids can be described on the basis of a single unique transducer function. In this paradigm, the nonsteroidal GABA$_A$ receptor modulators behave as partial agonists relative to neuroactive steroids.

The pharmacokinetic-pharmacodynamic correlations of benzodiazepines have been the subject of numerous studies in both animals and humans (Danhof and Mandema, 1992; for review, see Laurijssens and Greenblatt, 1996), but the predictive value of the proposed models seems to be limited. To date, there is an increasing interest in the development of mechanism-based PK/PD models because they allow the prediction of drug effects in vivo in a strict, quantitative manner on the basis of results obtained in in vitro test systems. These models not only provide a scientific basis for the prediction of drug effects in humans on the basis of results obtained in animal studies but also allow a mechanistic understanding for observed interindividual variability in drug response (Van der Graaf and Danhof, 1997).

The need for mechanism-based modeling is illustrated by the difficulty of predicting the in vivo intrinsic activity of benzodiazepine receptor partial agonists in humans on the basis of results obtained in preclinical investigations. For example, in humans, the new benzodiazepine Ro 46-2153 behaved as a full agonist, whereas it was selected from preclinical studies based on its partial agonist properties (Goggin et al., 2000).

In mechanism-based PK/PD models that are based on receptor theory, a separation is made between the drug-specific properties and the system-specific properties (Van der Graaf and Danhof, 1997). In previous investigations, the full parametric operational model of agonism (Black and Leff, 1983) has been applied successfully to synthetic opiates (Cox et al., 1998), adenosine A$_1$ agonists (Van der Graaf et al., 1997, 1999), and 5-hydroxytryptamine$_{1A}$ agonists (Zuideveld et al.,

ABBREVIATIONS: PK/PD, pharmacokinetic/pharmacodynamic; DMCM, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; HPLC, high-pressure liquid chromatography; CCV, constant coefficient of variation; FOCE, first-order estimation method with interaction; ORG 21465, 2β-3α-5α-3-hydroxy-2-(2,2-dimethylmorpholin-4-yl)-pregnan-11,20-dione; ORG 20599, 2β-3α-5α-21-chloro-3-hydroxy-2-(4-morpholinyl)-pregnan-20-one.

2001). A characteristic feature of the operational model of agonism is that it contains a specific expression for the maximum effect that is achievable in the system. However, attempts to develop a mechanism-based PK/PD model for the effects of benzodiazepines have been only partially successful, which can be explained by the fact that in the studies reported to date, no saturation in the stimulus-response relationship has been observed (Tuk et al., 1999). Recently, however, significantly higher EEG effects have been observed for neuroactive steroids compared with benzodiazepines, indicating that with benzodiazepines the system maximum has not been reached (Visser et al., 2002a,b). Similar to benzodiazepines, neuroactive steroids are selective and potent modulators of GABA$_A$ receptor function (for review, see Lambert et al., 1995). Benzodiazepines and other ligands for the benzodiazepine site exert their effects through allosteric modulation of the GABA$_A$ receptor, thereby enhancing the actions of endogenous GABA (Sigel and Baur, 1988). Neuroactive steroids have dual effects at the GABA$_A$ receptor. At nanomolar concentrations neuroactive steroids potentiate the effect of GABA, whereas at micromolar concentrations they can directly activate the GABA$_A$ receptor (Cottrell et al., 1987).

Interestingly, all investigated neuroactive steroids (alphaxalone, pregnanolone, ORG 20599, and ORG 21465) exhibited biphasic concentration-effect relationships in vivo. At low concentrations the EEG effect increased from baseline to a maximum value that was the same for each neuroactive steroid and approximately 2 to 3 times higher than the maximum observed for the benzodiazepine displaying the highest intrinsic activity (diazepam). At higher concentrations of neuroactive steroids the effect decreased under the baseline toward isoelectric EEG. Based on these observations, a new mechanism-based PK/PD modeling approach was proposed, which features a parameterized biphasic stimulus response. For alphaxalone the biphasic stimulus-response relationship was successfully characterized by a parabolic function (Visser et al., 2002b). Next, it was shown that a single and unique stimulus-response relationship characterizes the effects of an array of different synthetic neuroactive steroids showing large differences in potency but not in intrinsic efficacy at the GABA$_A$ receptor (Visser et al., 2002a).

In the present investigation, the mechanism-based PK/PD model for neuroactive steroids has been applied to the EEG effects of various nonsteroidal GABA$_A$ receptor modulators. The EEG effects and the plasma concentrations of six benzodiazepines (diazepam, flunitrazepam, midazolam, clobazam, oxazepam, and bretazenil), an imidazopyridine (zolpidem), a cyclopyrrolone (zopiclone), and a β-carboline (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, DMCM) were investigated in rats upon 5-min intravenous infusion. In addition, receptor binding studies were performed to obtain in vitro estimates of the affinity and the intrinsic efficacy on the basis of the so-called "GABA-shift" properties (Wood et al., 1983) at the GABA$_A$ receptor.

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 [289 ± 33 g (mean ± S.D.); Broekman Breeding Facilities, Someren, The

Netherlands] were used in this investigation. 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 (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 (Visser et al., 2002b). 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% polyvinyl pyrrolidone 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.

Drugs and Dosages. Midazolam, oxazepam and diazepam were purchased from Duchefa Pharma BV (Haarlem, The Netherlands). Clobazam, flunitrazepam, zolpidem, and DMCM were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). 4-Methyl-1-piperazinecarboxylic acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-β] pyrazin-5-yl ester (zopiclone) was purchased from Tocris Cookson Inc. (Bristol, UK). Bretazenil was kindly donated by F. Hoffmann-La Roche (Basel, Switzerland). Midazolam and zolpidem were dissolved in 250 μl of saline with equimolar hydrochloric acid, and the other compounds were dissolved in 100 μl of dimethylacetamide. Rats were randomly assigned to treatment groups ($n = 6$–9) that received 9.4 ± 0.3 mg · kg^{-1} diazepam, 2.2 ± 0.07 mg · kg^{-1} flunitrazepam, 18.0 ± 0.9 mg · kg^{-1} clobazam, 4.7 ± 0.1 mg · kg^{-1} midazolam, 4.0 ± 0.1 mg · kg^{-1} zolpidem, 8.9 ± 0.1 mg · kg^{-1} oxazepam, 3.9 ± 0.1 mg · kg^{-1} zopiclone, 2.9 ± 0.1 mg · kg^{-1} bretazenil, 0.81 ± 0.03 mg · kg^{-1} DMCM, or vehicle in a 5-min zero-order infusion.

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. 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 an 80486 computer (Intel BV, Sassenheim, The Netherlands) and stored on hard disk for off-line analysis. After recording the EEG baseline for 45 min, a zero-order intravenous infusion of one of the compounds was administered to the conscious and freely moving rats using an infusion pump (BAS Bioanalytical Systems Inc., West Lafayette, IN). For each 5-s epoch, quantitative EEG parameters were obtained off-line by Fast Fourier transformation with a user-defined 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 sampled was kept equal to 1.8

ml during each experiment. Bretazenil and clobazam samples were immediately hemolyzed in MilliQ water, to which NaF had been added to stop esterase activity. For the other compounds, the blood samples were heparinized and centrifuged at 5000 rpm for 15 min for plasma collection. Blood and plasma samples were stored at −20°C until high-pressure liquid chromatographic (HPLC) analysis.

HPLC Analysis. The blood or plasma concentrations of the compounds were determined by a specific HPLC assay with UV detection (slightly modified from Mandema et al., 1991a; Hoogerkamp et al., 1996; Cleton et al., 1999). The procedure was as follows. The blood/plasma samples were diluted with 0.5 ml of 0.1 M NaOH, 50 μl of an internal standard 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 4500g. 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 μl of mobile phase of which 40 μl was injected into the HPLC system. A mixture of 25 mM phosphate buffer and acetonitrile was used as mobile phase for midazolam [50:50 (v/v), pH 7.5], flunitrazepam [58:42 (v/v), pH 5.0], zolpidem [60:40 (v/v), pH 7.0], clobazam [60:40 (v/v), pH 7.0], and DMCM [60:40 (v/v), pH 7.0]. A mixture of 25 mM acetate buffer and acetonitrile was used as mobile phase for bretazenil [55:45 (v/v), pH 5.0], oxazepam [60:40 (v/v), pH 3.8], zopiclone [56:44 (v/v), pH 5.0], and diazepam [53:47 (v/v), pH 3.8]. The chromatographic system consisted of a M-45 solvent delivery pump, a WISP 717 automatic injector (all of Millipore Corporation, Bedford, MA), a column (150 × 4.6 mm C18, 5 μm; Alltech BV, Breda, The Netherlands) equipped with a hand-packed C18 guard column (20 × 2 mm i.d.), and a spectroflow 757 Kratus UV detector (Spark Holland BV, Emmen, The Netherlands). Bretazenil, clobazam, diazepam, DMCM, flunitrazepam, midazolam, oxazepam, and zolpidem were detected at wavelengths of 235, 222, 227, 278, 218, 222, 230, and 215 nm, respectively. The detector output was recorded using a Shimadzu C-R3A integrator (Shimadzu, Hertogenbosch, The Netherlands). In some cases the analytical procedure was slightly modified (Berrueta et al., 1992). The zopiclone and flunitrazepam plasma samples were diluted with 0.5 ml of 0.2 M borate buffer, pH 8.0, and 0.5 ml of 1 M borate buffer, pH 9.5, respectively. The oxazepam samples were diluted with 0.5 ml of 0.1 M phosphate buffer, pH 5.0, followed by methanol extraction using octadecyl 3-ml solid phase extraction columns (Bakerbond; Baker BV, Deventer, The Netherlands) (Mawa et al., 1996). Zopiclone concentrations were measured using a PerkinElmer LC240 fluorescence detector (Beaconsfield, UK) at wavelengths for excitation of 300 nm and for emission of 470 nm (Foster et al., 1994).

Linear calibration curves were obtained in the range of 0.01 to 10 μg · ml^{-1} for each compound. Inter- and intraday variability and the extraction recovery were determined using two quality controls (0.3 and 9 μg · ml^{-1}). Limit of quantification for each compound, based on a 50-μl plasma (or 100-μl blood) sample, the inter- and intra-assay variability, and the extraction recovery were for bretazenil 0.025 μg · ml^{-1}, 8, 5, and 85%; for clobazam 0.013 μg · ml^{-1}, 20, 20, and 83%; for diazepam 0.025 μg · ml^{-1}, 9, 8, and 95%; for DMCM 0.01 μg · ml^{-1}, 15, 10, and 80%; for flunitrazepam 0.025 μg · ml^{-1}, 4, 4, and 98%; for midazolam 0.025 μg · ml^{-1}, 6, 6, and 110%; for oxazepam 0.10 μg · ml^{-1}, 5, 5, and 89%; for zolpidem 0.05 μg · ml^{-1}, 10, 9, and 96%; and for zopiclone 0.025 μg · ml^{-1}, 10, 7, and 86%, respectively.

Protein Binding. Plasma protein binding was determined ex vivo after administration of 5 mg · kg^{-1} (n = 3) and 10 mg · kg^{-1} (n = 3) zopiclone or 1 mg · kg^{-1} DMCM (n = 6). At two time points 2-ml blood samples were drawn. For the other benzodiazepines the degree of plasma protein binding was determined in vitro at two concentrations (n = 3). Freshly heparinized blood (2 ml) was spiked with the compound and placed in a water bath at 37°C for 30 min. After equilibration, two samples (100 μl) of the spiked blood were hemolyzed with 500 μl of MilliQ water. Subsequently, the tubes were centrifuged for 10 min at 5000 rpm for plasma collection. From each

tube, two plasma samples of 50 μl were taken and the remaining plasma was centrifuged at 37°C (15 min, 2000g) using an ultrafiltration device (Centrifree; Millipore Corporation). Two samples of 100- to 400-μl ultrafiltrate were taken. The concentration of drug in the blood, plasma, and ultrafiltrate samples was measured by HPLC, according to the methods described above. The free fraction (f_u) was calculated by dividing the free concentration in ultrafiltrate by the total (bound and free) concentration in plasma.

In Vitro Pharmacology. The receptor binding affinity and the GABA-shift were determined in rat cortex homogenates in vitro. Briefly, the procedures of these assays were as follows: three groups of rats (n = 12–15) were used to obtain three brain homogenates. The rats were sacrificed by decapitation and the whole brain was excised. The homogenates were prepared using the method described by Mandema et al. (1991b), which was slightly modified. The cortex was removed from the whole brain and gently homogenized in 10 volumes of ice-cold "enriched" Tris buffer, pH 7.4, containing 50 mM Tris (Sigma-Aldrich), 120 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, and 1 mM MgCl$_2$ (all of Baker), in a Potter-Elvehjem homogenizer (10× at 4°C; Braun Potter S, Melsungen, Germany). The homogenates were centrifuged for 20 min at 45,000g at 4°C and the supernatant was discarded. The pellets were resuspended in 10 volumes of the enriched Tris buffer. This procedure was repeated four times. Protein concentration was measured using a DC protein assay (Bio-Rad BV, Veenendaal, The Netherlands). The homogenates (3 mg protein/ml) were stored in 1-ml fractions at −80°C until use.

The receptor binding characteristics (K_d and B_{max}) of the radioligand [^{3}H]flumazenil (specific activity 87.0 Ci/mmol; New England Nuclear 757, Hoofddorp, The Netherlands) were determined in saturation experiments in all three homogenates. Brain homogenate aliquots containing 1 mg of protein were incubated for 35 min at 37°C with increasing concentrations of the radioligand (0.5–330 nM) in the absence and presence of 50 μM GABA. Subsequently, for each compound, the GABA$_A$ receptor binding was determined by displacement of [^{3}H]flumazenil. Cortex homogenate aliquots containing 1 mg of protein were incubated for 35 min at 37°C with increasing concentrations of the compounds and a final assay concentration of 7 nM [^{3}H]flumazenil.

In both the saturation and the displacement experiments the efficacy of the various compounds was evaluated by examining the effect of 50 μM GABA on the receptor binding, the so-called GABA-shift. It has been shown that the ratio of the IC$_{50}$ values in the absence and in the presence of GABA is a reflection of the agonist efficacy of the compound (Wood et al., 1983). Thus, saturation and displacement experiments were carried out in the absence and presence of 50 μM GABA. In all experiments the nonspecific binding was determined in presence of 20 μM unlabeled flumazenil. In each tube, the final assay volume was 100 μl, including the ethanol concentration necessary to dissolve the compounds, fixed at 0.5% and the NaCl concentration at 1 M, to improve the assay performance. After incubation, the reaction was terminated by filtration through a presoaked GF/B filter (Whatman, Maidstone, UK) under mild suction using a harvester (Brandel Inc., Gaithersburg, MD). The filters were washed three times (or five times in saturation experiments) with 5 ml of ice-cold enriched Tris buffer. The filters were submerged in 5 ml of Emulsifier Safe scintillation fluid (PerkinElmer Life Sciences, Boston, MA) and vortexed. After incubation of 2 h, the radioactivity was measured with a Packard TriCarb 4640 liquid scintillation counter. Each experiment was performed in duplicate.

Pharmacokinetic-Pharmacodynamic Data Analysis. Pharmacokinetic compartmental analysis was performed by fitting of a two-compartment model to the concentration-time profiles of the compounds by use of the ADVAN3 TRANS4 subroutine within the nonlinear mixed effect modeling software package NONMEM (NONMEM project group, University of California, San Francisco, CA). The NONMEM program is based on a statistical model, which explicitly takes into account both interindividual variability and intraindividual residual error (Schoemaker and Cohen,

1996). The two-compartment model was selected for each compound on the basis of visual inspection of the model fits and the Akaike information criterion (Akaike, 1974). The pharmacokinetic parameters clearance (CL), intercompartmental clearance (Q), and the volumes of distribution of compartments 1 and 2 (V_1 and V_2) were estimated. The interindividual variability of these parameters was modeled according to the exponential equation:

$$P_i = \theta_i \cdot \exp(\eta_i) \qquad (1)$$

where θ_i is the population estimate for parameter P, P_i is the individual estimate, and ηi 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:

$$C_{mij} = C_{pij} \cdot (1 + \varepsilon_{ij}) \qquad (2)$$

where C_{pij} represents the jth plasma concentration for the ith 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 with interaction (FOCE) was used to estimate the population θ, ω^2, and σ^2. Individual parameter estimates were obtained in a Bayesian post hoc step and V_{dss} and half-life were calculated following standard procedures (Gibaldi and Perrier, 1982).

Individual parameter estimates were used to calculate individual blood/plasma concentrations at the times of the EEG measurements. For each compound, the individual concentration-effect curves thus obtained were fitted simultaneously to the Hill equation:

$$E = E_0 + \frac{\alpha \cdot C^{n_H}}{\mathrm{EC}^{n_H}_{50} + C^{n_H}} \qquad (3)$$

where E_0 is the no-drug response, α is the intrinsic activity, EC_{50} is the potency, and n_H is the slope factor. Interindividual variability for α and n_H was modeled using a proportional error model:

$$P_i = \theta_i \cdot (1 + \eta_i) \qquad (4)$$

and for the EC_{50} using an exponential error model (eq. 1). Similar to the pharmacokinetics, the residual variability in the pharmacodynamics was modeled as a CCV error according to eq. 2 and the FOCE was used for the estimation.

Mechanism-Based PK/PD Analysis. Subsequently, the concentration-effect data were analyzed by the recently proposed mechanism-based model for neuroactive steroids. In this model, the effect is thought to be a function of the stimulus induced by the drug-receptor binding (Tuk et al., 1999; Visser et al., 2002a,b). 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 (Fig. 2; Tuk et al., 1999; Visser et al., 2002a). A unique feature of this model is that the receptor activation process is drug-specific, whereas the stimulus-response relationship is system specific. Thus, the receptor activation can be different for different drugs. The stimulus-response relationship on the other hand is the same, regardless of the drug that is tested.

The characterization of the drug receptor interaction is based on receptor theory adjusted for application to in vivo responses (Kenakin, 1997; Visser et al., 2002a,b). In Fig. 2B is shown that in this approach, the drug-receptor interaction consists of a binding step where drug A binds to the receptor and an initial receptor activation step. The drug binding to the receptor forms a complex [AR]. This complex [AR] produces a stimulus depending on the efficacy (ϵ). Taking the binding and initial activation together, a concentration-stimulus relationship can be derived. In this model,
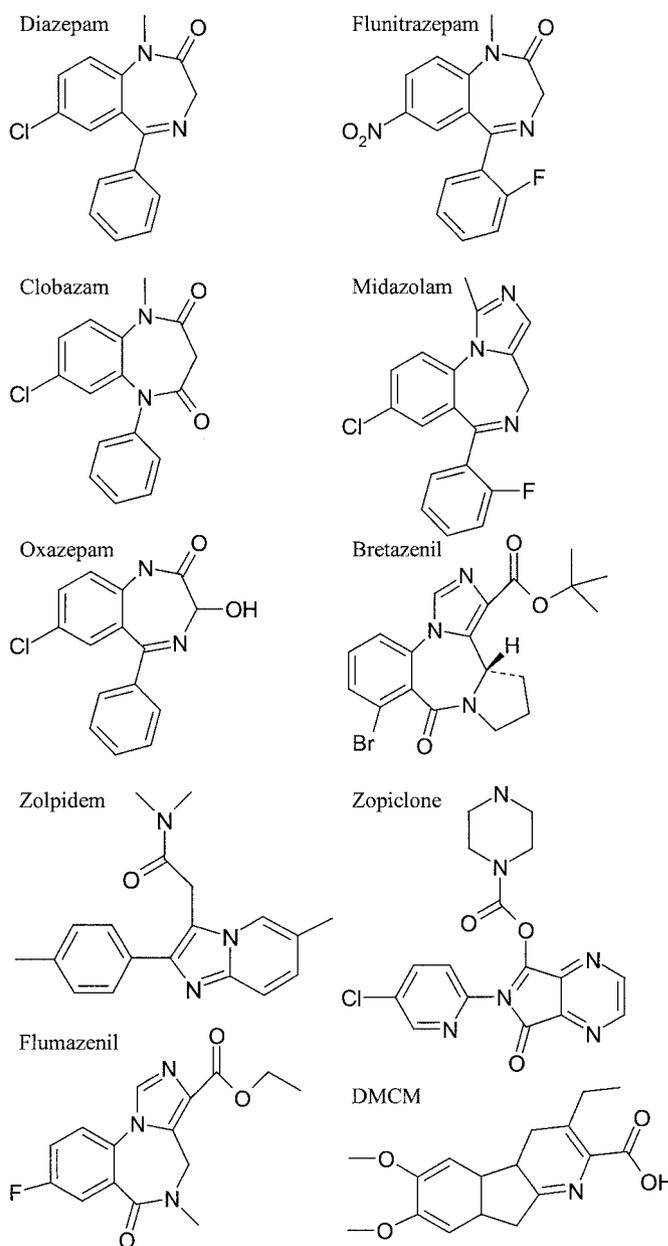

Fig. 1. Chemical structures of the benzodiazepines diazepam, flunitrazepam, midazolam, clobazam, oxazepam, and bretazenil; flumazenil; the imidazopyridine zolpidem; the cyclopyrrolone zopiclone; and the β-carboline DMCM.

the interaction with the receptor yields a stimulus S according to the formula:

$$S = \frac{e_{PD} \cdot C}{C + K_{PD}} \qquad (5)$$

where S is a function of the concentration (C), parameter K_{PD} represents in vivo estimated affinity ($K_A \cdot K_{AR}$), and e_{PD} is the in vivo estimated efficacy. In this theory $e_{PD} = \epsilon R_T$ in which ϵ is the stimulus generated upon activation of a single receptor and R_T is the total receptor concentration. Subsequently, this stimulus is propagated into the ultimate effect (E); its relation to the stimulus is given by a function f:

$$E = f(S) \qquad (6)$$

A

Drug-receptor interaction   **Stimulus-response relationship**
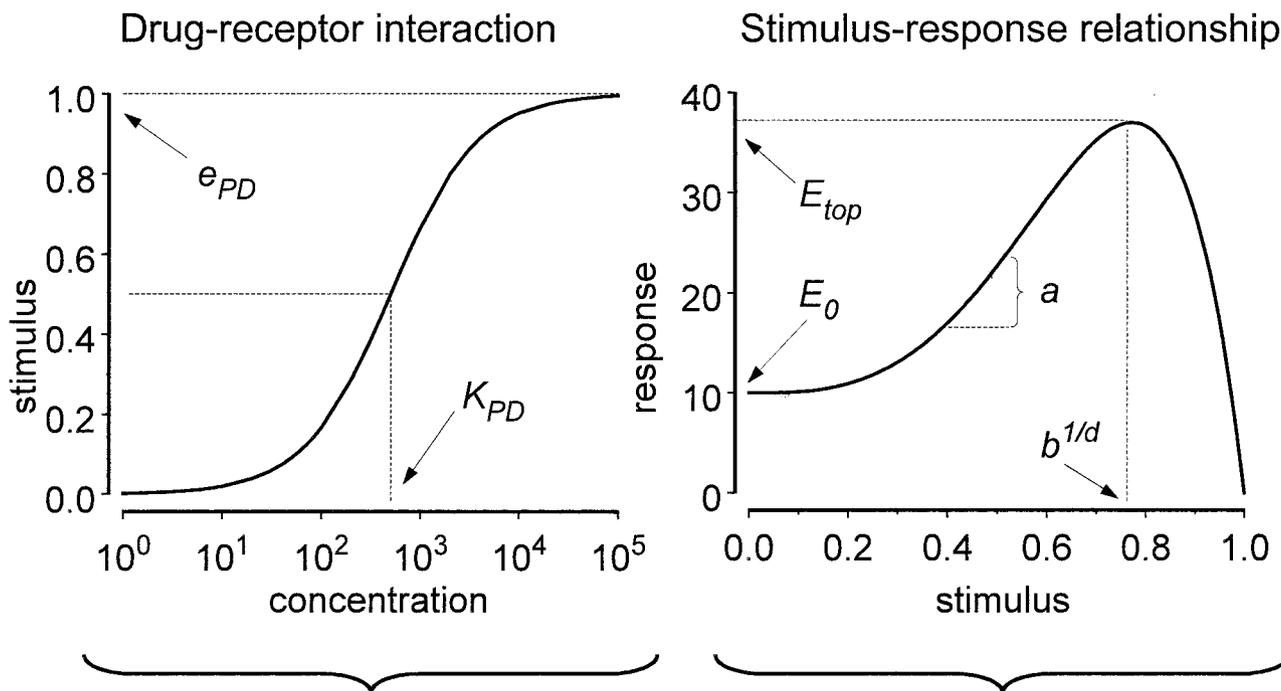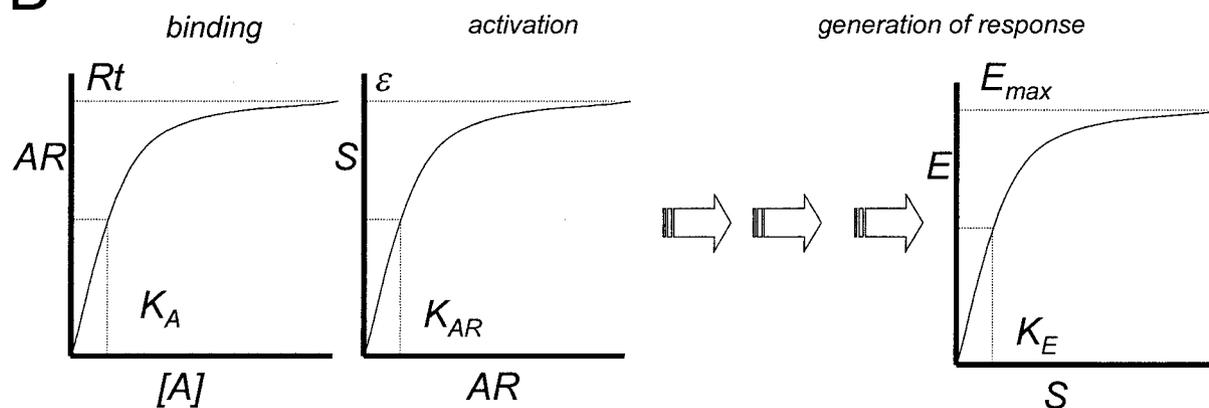
B

Fig. 2. A, proposed PK/PD model consists of two parts. The first part consists of a model for drug-receptor interaction (eq. 5), which is a hyperbolic function of the drug concentration producing a stimulus. K_{PD} is the concentration producing the half-maximal stimulus and e_{PD} is the maximal stimulus. The second part consists of a biphasic stimulus-response model, which is represented by a parabolic function (eq. 7). E_0 is baseline response, the top of the stimulus-response relationship is located at the value E_{top} and is obtained at the value $b^{1/d}$ and the slope of the parabolic function is determined by a. B, drug-receptor interaction (concentration-stimulus relationship) is a combination of the binding of the drug to the receptor, forming the drug-receptor complex (AR) and the activation of the drug-receptor complex into an initial stimulus with an efficacy (ϵ). Stimulus-response relationship contains all intermediate steps between the initial activation of the receptor and the ultimate EEG response.

In the analysis of the EEG effects of neuroactive steroids, 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):

$$E = E_{top} - a \cdot (S^d - b)^2 \tag{7}$$

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 characterizes the asymmetry of the parabola (Fig. 2). When no drug is present the EEG is equal to its baseline value (E_0). Equation 7 then reduces to the following:

$$E_0 = E_{top} - a \cdot b^2 . \tag{8}$$

Substituting eq. 8 in eq. 7, and rearranging yields:

$$E = E_0 - a \cdot ((S^d)^2 - 2 \cdot b \cdot S^d) , \tag{9}$$

where E_0 represents baseline value of the EEG. In the previous investigations a, b, and d have been determined for neuroactive steroids describing the full biphasic stimulus-response relationship. It was shown that a variation in baseline value (E_0) was reflected in the maximal achievable response in this system (E_{top}), via parameter a following the relationship (eq. 8):

$$a = A \cdot E_0 , \tag{10}$$

in which A is a linear proportionality constant.

In the analysis, the parameters determining the shape of the

stimulus-response relationship were fixed at the previously obtained values for synthetic neuroactive steroids with the corresponding interindividual variability [A = 9.2 (22%), b = 0.44 (7%), and d = 3.36 (−)], respectively (Visser et al., 2002a,b). To characterize the effects of inverse agonists, negative e_{PD} values must be estimated. For this reason, S^d was rewritten:

$$S^d = |S|^{d-1} \cdot S \qquad (11)$$

which is equal to S^d when $S > 0$, and equal to $-(-S)^d$ when $S < 0$.

For each GABA$_A$ receptor modulator the values of K_{PD} and e_{PD} were estimated. The e_{PD} was estimated relative to alphaxalone for which e_{PD} is 1 (i.e., maximal stimulus). Averaged amplitudes over 40 min of individual EEG recordings before infusion served as input for individual baseline values.

The interindividual variability of K_{PD} was modeled using an exponential error model, whereas interindividual variability of e_{PD} was modeled using a CCV error model. Similar to the pharmacokinetics, the residual variability in the pharmacodynamics was modeled as a CCV error according to eq. 2. The FOCE method with interaction was used to estimate the population θ, ω^2, and σ^2. 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.

In Vitro Data Analysis. The specific binding of [^{3}H]flumazenil was calculated by subtracting the nonspecific binding from the total binding measured in the saturation experiments. The [^{3}H]flumazenil concentrations versus the specific binding were fitted using the equation:

$$B = \frac{B_{max} \cdot L}{K_d + L} \qquad (12)$$

where B is the amount ligand bound at specific binding sites, B_{max} is the maximal amount of bound radioligand at specific binding sites, K_d is the ligand concentration at which 50% of the radioligand is bound to the receptor, and L is the free ligand concentration in the tube.

The displacement curves were described using the following equation:

$$B = B_0 - \frac{D_{max} \cdot C_d}{IC_{50} + C_d} \qquad (13)$$

where B_0 is the specific binding of the radioligand with no displacer present, D_{max} is the maximal displaced percentage, C_d is the concentration of displacer, and IC_{50} is the concentration of the displacer at 50% inhibition of the binding of the radioligand. Estimates for K_i values were derived from the IC_{50} values according to the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \dfrac{L}{K_d}} \qquad (14)$$

where K_i is the affinity of the displacer and L is the concentration of radioligand used in the displacement studies. The GABA-shift was calculated by dividing the IC_{50} in absence of GABA by the IC_{50} in presence of GABA. Nonlinear regression analysis for the saturation and displacement experiments was performed by implementing the equations in NONMEM.

Statistical Analysis. Goodness-of-fit was evaluated on basis of visual inspection of the model fits and the value of the objective function. Model selection was based on the Akaike Information Criterion (Akaike, 1974) and assessment of the parameter correlation. Statistical analysis was performed using one-way analysis of variance (ANOVA) 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 (GraphPad, San Diego, CA). All data are represented as mean ± S.E.M and the significance level was set to $\alpha < 0.05$.

Results

Pharmacokinetics and Pharmacodynamics. The concentration-time profiles of the various compounds were best described using a two-compartment pharmacokinetic model. The observed and predicted pharmacokinetic profiles are depicted in Fig. 3. The population pharmacokinetic parameter estimates for each GABA$_A$ receptor modulator and the corresponding inter- and intraindividual variability are summarized in Table 1. Terminal half-life was 41 ± 2 min for diazepam, 42 ± 4 min for flunitrazepam, 33 ± 2 min for clobazam, 32 ± 1 min for midazolam, 20 ± 1 min for zolpidem, 64 ± 3 min for oxazepam, 69 ± 3 min for zopiclone, 53 ± 9 min for bretazenil, and 20 ± 1 min for DMCM.

For each compound the protein binding was determined. For DMCM, no free fraction could be determined. Except for zopiclone and bretazenil, the blood-plasma ratio was determined for each compound. The fluorescence assay for zopiclone was not suitable for the analysis of blood samples, whereas for bretazenil, no plasma samples could be taken for stability reasons. Protein binding and blood/plasma concentration ratios are summarized in Table 1.

Concentrations were calculated at the time points of effect measurements and the resulting concentration-effect relationships were fitted to the Hill equation (eq. 3), yielding estimates for intrinsic activity (α), potency (EC$_{50}$), baseline (E_0), and slope (n_H). The observed concentration-effect relationships for each GABA$_A$ receptor modulator are shown in Fig. 4. The highest intrinsic activity was found for diazepam (10.6 ± 1.2 μV). Bretazenil showed an intrinsic activity of 1.2 ± 0.5 μV, whereas DMCM revealed a negative α of −0.8 ± 0.2 μV as depicted in Table 2. The intrinsic activity for DMCM and bretazenil could only be determined by fixing the individual baseline values, because the variation in baseline between the animals was larger than the intrinsic activity of DMCM and bretazenil itself. However, all individual profiles showed a decrease (DMCM) or increase (bretazenil) in EEG compared with vehicle treatment experiments. Statistical analysis showed that the intrinsic activity of diazepam was different from the intrinsic activity of zolpidem, oxazepam, zopiclone, bretazenil, and DMCM but not from midazolam, flunitrazepam, and clobazam ($p < 0.05$).

Mechanism-Based PK/PD Modeling. The concentration-effect relationships were simultaneously fitted to the mechanism-based model wherein the parameters A, b, and d of the stimulus-response relationship fixed to values determined using synthetic neuroactive steroids (Visser et al., 2002a,b). The model was able to successfully describe all individual concentration-effect relationships. The population prediction and individual predictions are shown in Fig. 4. Wide differences were observed in e_{PD} and K_{PD} as shown in Table 3. The values of the e_{PD} ranged from −0.27 (DMCM) to 0.54 (diazepam) and the values of the K_{PD} ranged from 0.41 ± 0 ng · ml^{-1} (bretazenil) to 392 ± 76 ng · ml^{-1} (clobazam). For all compounds, the population concentration-stimulus relationship with their standard error of K_{PD} and e_{PD} are shown in Fig. 5A. In this graph, the concentration-stimulus relationships of the neuroactive steroids (alphaxalone,
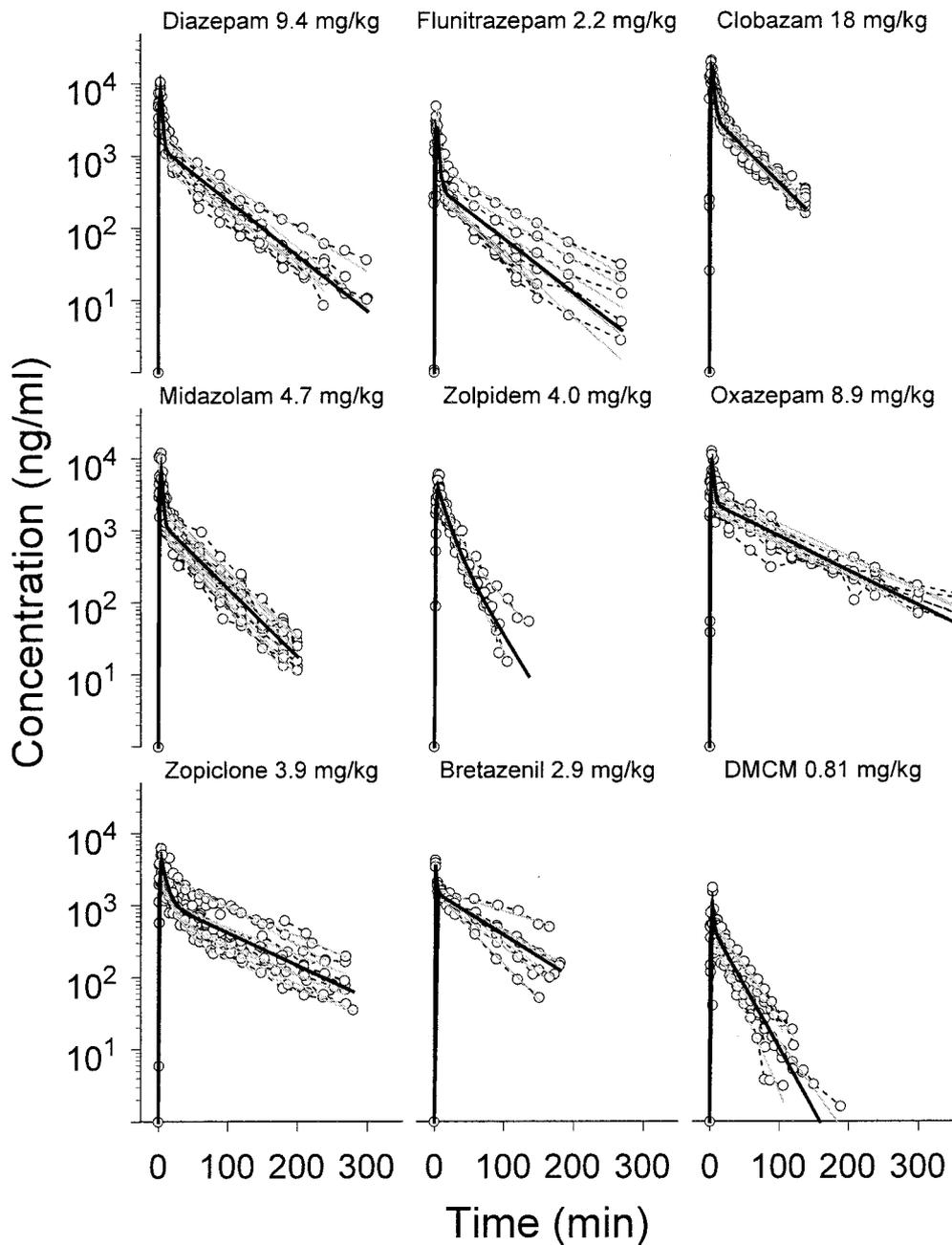
Fig. 3. Pharmacokinetics of the GABA$_A$ receptor modulators. Observed (open circles with dotted line), individual predicted (thin lines), and population predicted (thick lines) concentration-time profiles for all compounds. Time in minutes is depicted on the x-axis and the concentration is depicted on the y-axis on a logarithmic scale. The name of the compound and the dose are depicted in the graphs.

pregnanolone, ORG 20599, and ORG 21465) are included (Visser et al., 2002a). Interestingly, it was observed that the GABA$_A$ receptor modulators with little difference in their in vivo efficacy, show large differences in intrinsic activity (e.g., zolpidem versus midazolam), whereas bretazenil and DMCM had significant different estimates for in vivo efficacy, but showed marginal EEG effects.

In Fig. 5B, the stimulus-response relationship of the benzodiazepines, imidazopyridine, cyclopyrrolone, and β-carboline are superimposed on the stimulus-response relationship that was found for the neuroactive steroids (Visser et al., 2002a). This analysis shows that benzodiazepines and other allosteric GABA$_A$ receptor modulators differ from neuroactive steroids both with respect to affinity and intrinsic efficacy at the GABA$_A$ receptor.

In Vitro Pharmacology. In three homogenates, the K_d and B_{max} of [^{3}H]flumazenil were estimated using NONMEM. The K_d and B_{max} of flumazenil in absence of GABA were 10.6 ± 2.7 nM (51%) and 111 ± 10 fmol $\cdot$ mg^{-1} protein (18%), respectively. The K_d and B_{max} were not significantly affected by the presence of 50 μM GABA [10.4 ± 1.2 nM (16%) and 124 ± 5 fmol $\cdot$ mg^{-1} protein (15%), respectively]. This is consistent with the fact that flumazenil acts as a competitive antagonist at the GABA$_A$ receptor with an intrinsic efficacy that is not significantly different from zero.

Subsequently, in displacement studies, for each compound the IC$_{50}$ for displacement of [^{3}H]flumazenil was measured in the presence and absence of 50 μM GABA. The displacement studies were performed in duplicate in three homogenates. All curves were successfully described by eq. 13. The value

TABLE 1
Population pharmacokinetic parameter estimates and standard error of estimate ($\theta \pm$ S.E.) for CI, Q, V_1, and V_2 with the corresponding interindividual coefficient of variation (CV%) in parentheses

The intraindividual residual variation is depicted in the res-CV column. The free fraction of benzodiazepines in plasma (f_u) and the blood-plasma ratio (B/P) are depicted in the last two columns.

	CL	Q	V_1	V_2	res-CV	f_u	B/P
	$ml \cdot min^{-1} \cdot kg^{-1}$		$l \cdot kg^{-1}$		%	%	
Diazepam	79.5 ± 6.9(20)	125 ± 13 (<1)	0.36 ± 0.10(65)	2.65 ± 0.18(20)	(29)	10.0 ± 0.1[a]	1.4 ± 0.1[a]
Flunitrazepam	58.6 ± 8.3(32)	75.3 ± 12 (<1)	0.33 ± 0.05(24)	1.81 ± 0.17(16)	(34)	26.6 ± 0.3	1.6 ± 0.1[a]
Clobazam	69.5 ± 4.0(11)	88.9 ± 14 (<1)	0.51 ± 0.13(57)	1.66 ± 0.18(17)	(28)	37.7 ± 3.8	1.8 ± 0.7
Midazolam	50.4 ± 5.0(23)	80.1 ± 12 (1)	0.23 ± 0.05(54)	1.32 ± 0.14(17)	(30)	6.6 ± 1.5	1.0 ± 0.1
Zolpidem	52.6 ± 3.8(14)	10.4 ± 3.7 (<1)	0.78 ± 0.07(15)	0.22 ± 0.15(1)	(25)	4.2 ± 0.1	1.7 ± 0.3
Oxazepam	34.0 ± 3.2(24)	172 ± 53 (<1)	0.42 ± 0.16(78)	2.23 ± 0.31(27)	(31)	11.7 ± 2.1	1.2 ± 0.1[a]
Zopiclone	27.5 ± 3.8(42)	57.7 ± 11 (34)	0.67 ± 0.16(65)	1.44 ± 0.12(26)	(21)	16.6 ± 0.8	N.D.
Bretazenil	25.0 ± 3.8(37)	207 ± 19 (<1)	0.52 ± 0.04(<1)	1.15 ± 0.03(<1)	(9)	9.8 ± 0.3	N.D.
DMCM	53.3 ± 7.7(34)	158 ± 67 (<1)	0.37 ± 0.21(99)	0.78 ± 0.15(20)	(27)	<0.1	0.45 ± 0.01

N.D., not determined.
[a] Taken from Hoogerkamp et al. (1996)

for K_i was calculated using eq. 14. The in vitro K_i values and the GABA-shifts are summarized in Table 3. In line with the in vivo estimates of e_{PD}, the GABA-shifts of the compounds do not show large differences except for bretazenil and DMCM.

In Vitro-in Vivo Correlations. Figure 6 shows a plot of the values of the K_i determined in vitro in the presence of GABA versus the in vivo K_{PD}. When using total concentrations, a linear relationship was observed between the logarithm of the K_i and the K_{PD}: $\log(K_{PD}) = 1.15 \pm 0.22 \cdot \log(K_i) - 0.003 \pm 0.37$ ($r = 0.85$) (A). This relationship was improved by correcting the values of K_{PD} for protein binding (B). The relationship between K_{PD} for the free (unbound) concentrations ($K_{PD,unb}$) and K_i was $\log(K_{PD,unb}) = 1.03 \pm 0.12 \cdot \log(K_i) - 0.86 \pm 0.02$ ($r = 0.93$). Because no protein binding for DMCM could be determined, the value for K_{PD} of DMCM was not corrected for protein binding. For neuroactive steroids, the in vitro values, representing the IC$_{50}$ for [^{35}S]t-butylbicyclophosphorothionate inhibition, were taken from Anderson et al. (1997) and the values for the in vivo K_{PD} were from Visser et al. (2002a).

The relationship between the logarithm of the GABA-shift and estimated in vivo efficacy e_{PD} is shown in Fig. 7. The solid line represents the linear relationship that was observed using the data obtained in this investigation: $e_{PD} = 1.96 \pm 0.19 \cdot \log(\text{GABA-shift}) - 0.11 \pm 0.03$ ($r = 0.97$). The dotted line represents the relationship that includes the GABA-shifts for pregnanolone and ORG 20599 from the literature (McCauley et al., 1995; Van Rijn et al., 1999), which is described by the equation $e_{PD} = 1.75 \pm 0.21 \cdot \log(\text{GABA-shift}) - 0.14 \pm 0.05$ ($r = 0.95$). On the basis of the information in Fig. 7 and the shape of the unique biphasic stimulus-response relationship, it is in principle possible to predict for each compound the maximum EEG effect in vivo on the basis of information on the GABA-shift from the in vitro receptor binding assay. This relationship is complex as is shown in Fig. 8.

Discussion

In the present investigation, the mechanism-based PK/PD model for synthetic neuroactive steroids has been applied to the in vivo EEG effects of six benzodiazepines, an imidazopyridine, a cyclopyrrolone, and a β-carboline. The compounds were selected on the knowledge that they display different intrinsic activity in vivo (Mandema et al., 1991b, 1992). These differences in intrinsic activity (α) and potency were confirmed in the present investigation by the descriptive analysis using the Hill equation. It was shown that benzodiazepines in general have a similar intrinsic activity, although there is a tendency for slightly lower values for flunitrazepam, midazolam, and clobazam relative to diazepam. This difference, however, did not have statistical significance. This is in agreement with several other reports where one of these four benzodiazepines has been reported to have the highest intrinsic activity in vivo (Mandema et al., 1991; Facklam et al., 1992). The nonbenzodiazepines have a lower intrinsic activity. It has been suggested that these compounds have pharmacological profiles different from benzodiazepines due to either differential affinity for different GABA$_A$ receptor subtypes or partial agonistic properties (Depoortere et al., 1986; Ueki et al., 1987). Bretazenil showed a marginal EEG effect, which is in agreement with the results of previous investigations where it was shown that bretazenil has an intrinsic activity that is 20% of the intrinsic activity observed with midazolam (Mandema et al., 1992). DMCM, reported a full inverse agonist (Kemp et al., 1987), showed a small but statistically significant negative EEG effect (Massotti et al., 1985). The potencies (EC$_{50}$) of the various compounds were in the same range and order as reported previously (Mandema et al., 1991, 1992).

In the analysis with the mechanism-based PK/PD model that was developed for the biphasic concentration-effect relationship neuroactive steroids, it was shown that this model is generally applicable for the description and prediction of the EEG effects of benzodiazepines and other GABA$_A$ receptor modulators. An interesting feature of the model is that it allows estimation of the absolute intrinsic efficacy at the GABA$_A$ receptor, rather than the intrinsic efficacy relative to other benzodiazepines, because with neuroactive steroids the physiological maximum of the system (i.e., isoelectric EEG) is reached. In addition, the model is able to estimate the intrinsic efficacy of a negative GABA$_A$ receptor modulator. The findings of the present analysis show that all investigated compounds behave as partial (or inverse) agonists at the GABA$_A$ receptor with intrinsic efficacies varying between −0.27 and 0.54.

The observed and predicted shape of the stimulus-response relationship for benzodiazepines (Fig. 7) is found to be simi-
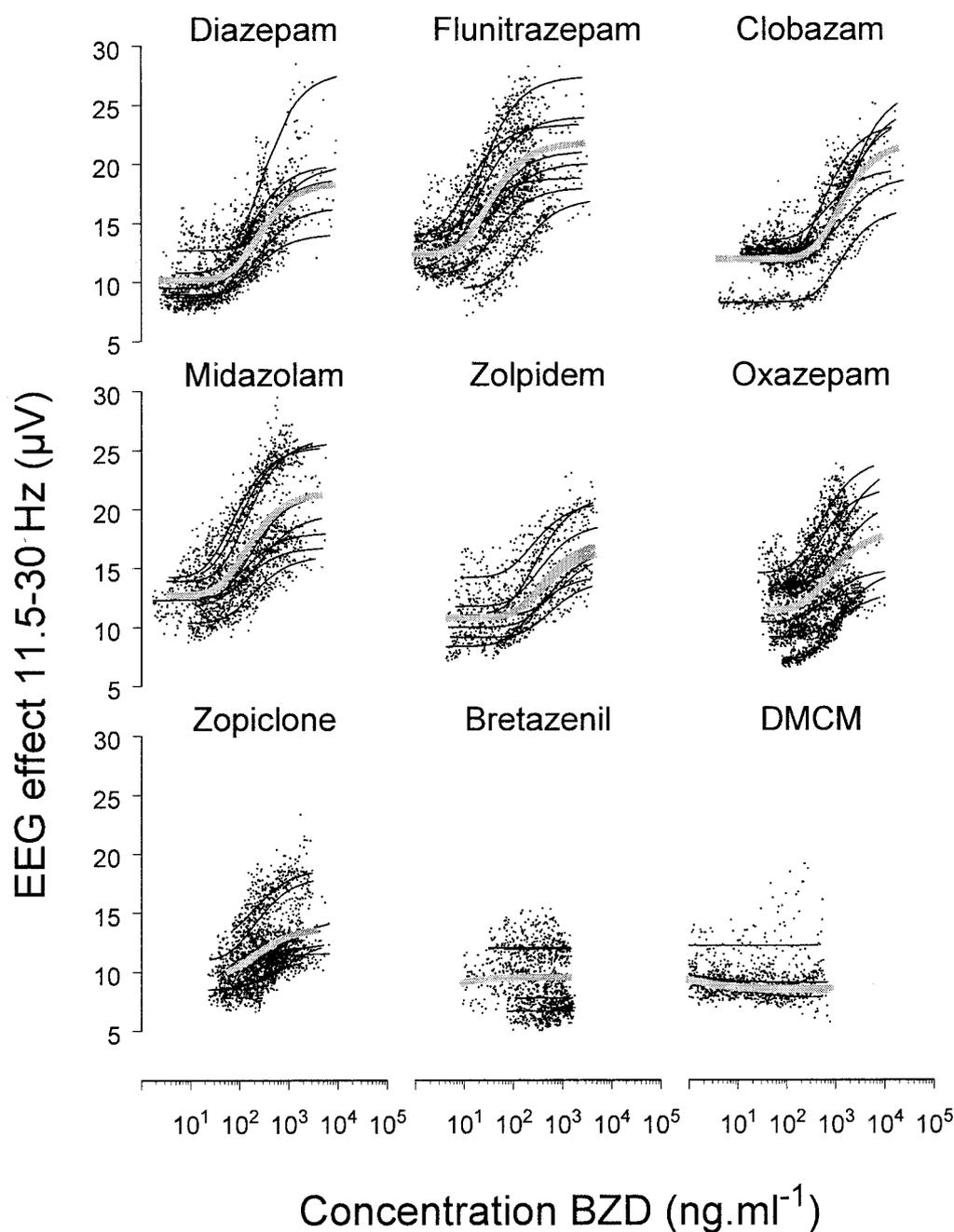
Fig. 4. Pharmacodynamics of the $GABA_A$ receptor modulators. Observed (dots), individual predicted (thin lines) and population-predicted (thick lines) concentration-effect profiles for 9.4 ± 0.3 mg·kg⁻¹ diazepam, 2.2 ± 0.07 mg·kg⁻¹ flunitrazepam, 18.0 ± 0.9 mg·kg⁻¹ clobazam, 4.7 ± 0.1 mg·kg⁻¹ midazolam, 4.0 ± 0.1 mg·kg⁻¹ zolpidem, 8.9 ± 0.1 mg·kg⁻¹ oxazepam, 3.9 ± 0.1 mg·kg⁻¹ zopiclone, 2.9 ± 0.1 mg·kg⁻¹ bretazenil, and 0.81 ± 0.03 mg·kg⁻¹ DMCM. The concentration (nanograms per milliliter) of the compounds is depicted on the x-axis on a logarithmic scale. The effect as amplitude in β-frequency range (microvolts) is depicted on the y-axis. The predicted lines were obtained using the mechanism-based PK/PD model (eq. 9) and were not visually different from the predictions of the Hill equation.

lar to the stimulus-response relationship observed by Tuk et al. (1999) in the sense that no saturation at the higher stimulus intensities is reached. In that investigation, the stimulus-response relationship was characterized as a monotonically increasing function. In the present investigation, it is shown that this stimulus-response relationship coincides with the initial part of the biphasic stimulus-response relationship of synthetic neuroactive steroids. This shows that benzodiazepines and other nonsteroidal $GABA_A$ receptor modulators differ from neuroactive steroids solely with respect to the interaction at the $GABA_A$ receptor in the sense that they behave as partial agonists. The present analysis shows that despite the very different in vivo concentration-effect relationships for both classes of compounds the only difference is the degree of maximal receptor activation.

To evaluate the validity of the present modeling, in vitro-in vivo correlations were explored, both with respect to potency and intrinsic efficacy. The binding affinity of the nonsteroidal $GABA_A$ receptor modulators was determined in rat brain homogenates in vitro in the presence and absence of GABA (Wood et al., 1983). The binding constant K_i in the presence of 50 μM GABA was taken as the most representative for the in vivo binding affinity. Between the compounds wide differences in in vivo binding affinity (K_{PD}) were observed with values ranging from 0.41 ng·ml⁻¹ for bretazenil to 392 ng·ml⁻¹ for clobazam. Interestingly, the values of the K_{PD} for flunitrazepam, midazolam, oxazepam, and clobazam are remarkably similar to the values reported by Tuk et al. (1999). In the analysis of the in vitro-in vivo correlation for potency both total and free (i.e., unbound) plasma concentra-

TABLE 2

Population pharmacodynamic parameter estimates and standard error of estimate ($\theta \pm$ S.E.) for intrinsic activity (α), potency (EC$_{50}$), Hill slope (n_H), and baseline (E_0) with the corresponding interindividual coefficient of variation (CV%) in parentheses

Intraindividual residual variation (res-CV) is depicted in the last column.

	α	EC$_{50}$	Hill	E_0	res-CV
	μV	$ng \cdot ml^{-1}$		μV	%
Diazepam	10.6 ± 1.2 (27)[a]	373 ± 97 (56)	1.17 ± 0.17 (23)	9.9 ± 0.7 (17)	(11)
Flunitrazepam	9.3 ± 0.7 (19)	35.5 ± 10 (78)	1.23 ± 0.11 (22)	11.8 ± 0.5 (13)	(8)
Midazolam	9.2 ± 1.1 (33)	161 ± 33 (48)	1.13 ± 0.08 (<1)	12.1 ± 0.5 (9)	(9)
Clobazam	7.5 ± 0.5 (16)	1080 ± 217 (49)	2.35 ± 0.32 (34)	12.0 ± 0.6 (13)	(7)
Zolpidem	5.8 ± 0.7 (26)	290 ± 74 (64)	2.2 ± 0.7 (46)	10.5 ± 0.7 (17)	(9)
Oxazepam	4.9 ± 0.6 (37)	612 ± 119 (53)	2.74 ± 0.38 (32)	11.5 ± 0.9 (21)	(6)
Zopiclone	3.8 ± 0.6 (35)	179 ± 48 (73)	2.94 ± 0.73 (58)	9.2 ± 0.4 (16)	(10)
Bretazenil	1.2 ± 0.5 (99)	1.3 ± 0 (—)	0.62 ± 0.12 (—)	9.8 ± 1.2 (fixed)	(11)
DMCM	-0.8 ± 0.2 (45)	0.95 ± 2.3 (520)	1.77 ± 0.30 (0)	10.3 ± 1.5 (fixed)	(13)

—, fixed at zero, thus no interindividual variation.

[a] Different from the intrinsic activity of zolpidem, oxazepam, zopiclone, bretazenil, and DMCM but not from midazolam, flunitrazepam, and clobazam ($p < 0.05$).

TABLE 3

In vivo and in vitro estimates for affinity and efficacy of benzodiazepines

In vitro: averaged (mean $\pm$ S.E.M., $n = 3$) estimates of affinity (K_i, in the absence of GABA) and GABA-shift (IC$_{50, -GABA}$/IC$_{50, +GABA}$). Each determination was carried out in duplicate. In vivo: population pharmacodynamic parameter estimates and standard error of estimate ($\theta \pm$ S.E.) for K_{PD} and e_{PD} with the corresponding interindividual coefficient of variation (CV%) in parentheses. Intraindividual residual variation of the in vivo data (res-CV) is depicted in the last column.

	In Vitro		In Vivo		res-CV
	K_i	GABA-shift	K_{PD}	e_{PD}	
	$ng \cdot ml^{-1}$		$ng \cdot ml^{-1}$		%
Diazepam	15.0 ± 1.99[a]	ND	75.1 ± 12 (36)	0.54 ± 0.03 (8)	(11)
Flunitrazepam	9.8 ± 0.33	1.63 ± 0.05	9.5 ± 2.7 (79)	0.50 ± 0.01 (0.3)	(8)
Midazolam	9.7 ± 2.7	1.70 ± 0.40	41.2 ± 6.0 (40)	0.50 ± 0.02 (7)	(9)
Clobazam	328 ± 52	1.67 ± 0.25	392 ± 76 (50)	0.53 ± 0.02 (11)	(7)
Zolpidem	30.7 ± 8.1	1.64 ± 0.09	120 ± 24 (52)	0.48 ± 0.02 (3)	(9)
Oxazepam	113 ± 16	1.49 ± 0.12	224 ± 49 (61)	0.47 ± 0.02 (14)	(7)
Zopiclone	105 ± 17	1.26 ± 0.04	65.1 ± 29 (87)	0.42 ± 0.01 (10)	(10)
Bretazenil	0.4 ± 0.01	1.15 ± 0.01	0.41 ± 0 (—)	0.29 ± 0.04 (32)	(13)
DMCM	14.2 ± 4.3	0.69 ± 0.20	0.60 ± 0.6 (204)	-0.27 ± 0.01 (12)	(11)

—, fixed at zero, thus no interindividual variation.

[a] From Hoogerkamp et al. (1996).

tions were considered. Statistically significant linear correlations between the in vitro K_i and the estimated in vivo binding affinity K_{PD} were observed (Fig. 6). Correction for the free fractions in plasma resulted in a statistically significant increase of the correlation coefficient ($p < 0.001$), indicating that free plasma concentrations may be the most representative for concentrations at the site of action and the in vivo drug effect. Similar observations have been reported for benzodiazepines (Greenblatt et al., 1983; Arendt et al., 1987; Mandema and Danhof, 1992; Hoogerkamp et al., 1996) and neuroactive steroids (Visser et al., 2002a). For DMCM no free fraction could be determined in the ultrafiltrate despite a sufficiently low detection limit of the assay and might indicate a methodological error (e.g., sticking of DMCM to the membrane of the ultrafiltration device) or that protein binding is not an important factor for the pharmacodynamics of DMCM.

The so-called GABA-shift is a well established parameter to estimate the intrinsic efficacy of benzodiazepines and neuroactive steroids in vitro (Wood et al., 1983). Typically, rather small values of the GABA-shift are observed for benzodiazepines, whereas the values are typically larger for neuroactive steroids. In the present investigations, values for the GABA-shift varying between 0.69 ± 0.2 for DMCM and 1.70 ± 0.4 for midazolam were observed. These values are in the same range as reported by others. It should be realized, however, that considerable differences in the values of the GABA-shift between laboratories have been reported. This parameter may be relatively sensitive to the experimental conditions and the radioligand that has been used in the

characterization of the binding range (Wood et al., 1983; Petersen et al., 1984; Kemp et al., 1987; Dawson and Poretski, 1989; Mehta and Shank, 1995; Van Rijn et al., 1999). In the present investigation [³H]flumazenil has been used as a radioligand in the estimation of the GABA-shift for nonsteroidal GABA$_A$ receptor modulators. Flumazenil has no intrinsic efficacy itself, which facilitates estimation of the GABA-shift. Estimation of the GABA-shift for neuroactive steroids on the other hand is more complex. Because no direct ligand is available, the use of a ligand with positive or negative intrinsic efficacy is required to assess the GABA-shift, which makes interpretation more difficult. This may explain part of the variability in the values that have been reported.

A highly significant linear correlation was observed between the values of the in vivo intrinsic efficacy parameter e_{PD} and the log GABA-shift. In addition, the relationship between the log GABA-shift and the in vivo efficacy enabled the prediction of the "maximal" EEG response (Fig. 8). This relationship between the log GABA-shift and predicted intrinsic activity in vivo was found to be nonlinear with a shallow part at low stimulus intensities in both directions and a steeper part at higher stimulus intensities. The relationship between the log GABA-shift and intrinsic activity predicts that compounds with a GABA-shift higher than 2 will show biphasic EEG effects in rats. For pregnanolone, a GABA-shift of 2.3 has been reported (McCauley et al., 1995) and for ORG 20599, a 3.5-fold binding enhancement was observed in the presence of GABA (Van Rijn et al., 1999),
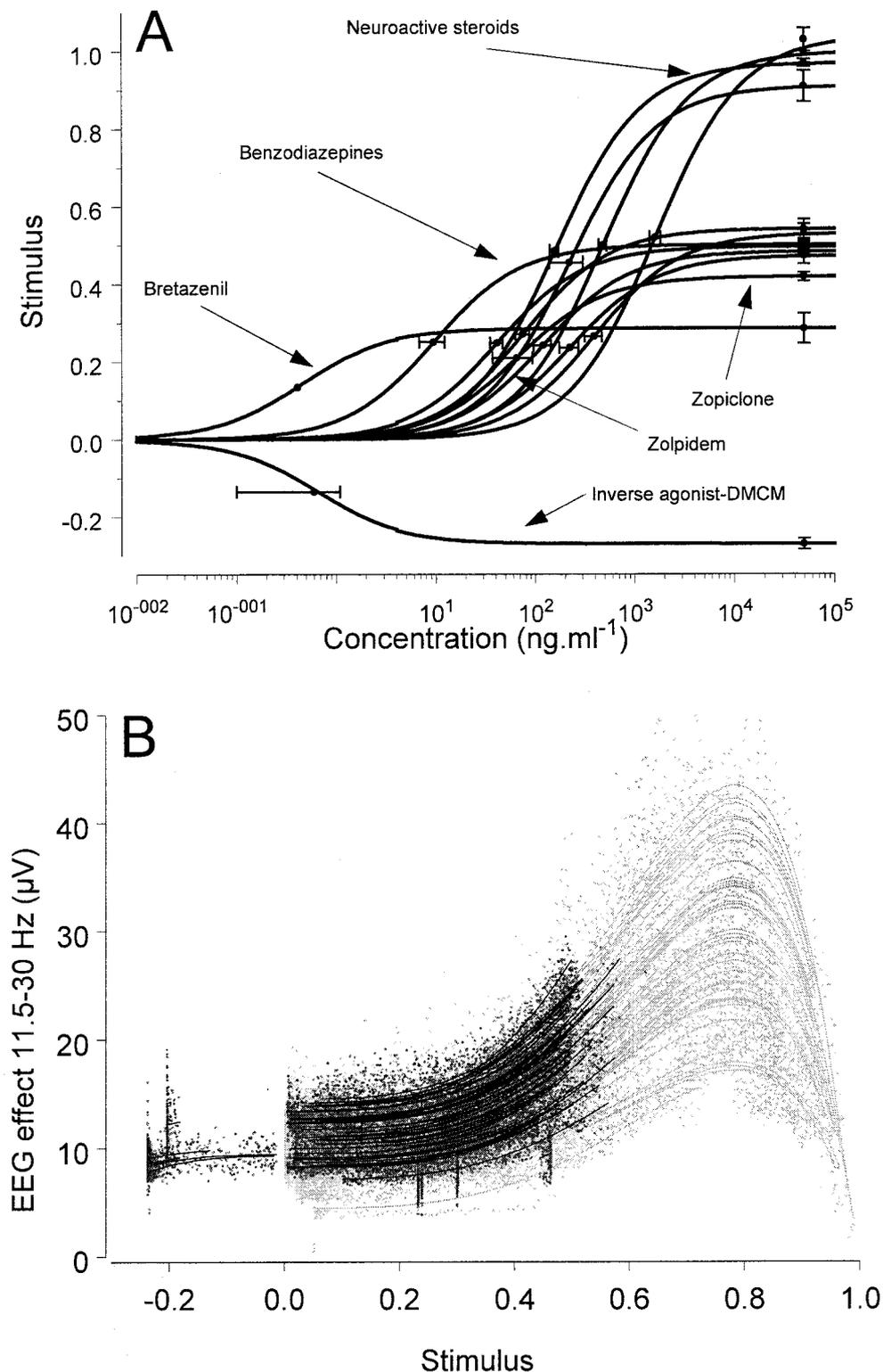
Fig. 5. Mechanism-based modeling. A, drug-receptor interaction. The relationship between concentration and stimulus for the $GABA_A$ receptor modulators. Concentration (nanograms per milliliter) is depicted on the x-axis in logarithmic scale and the stimulus is depicted on the y-axis. The neuroactive steroid concentration-stimulus relationships were taken from Visser et al. (2002a). B, stimulus-effect relationship. The stimulus-effect relationship of all compounds and for all individual rats, superimposed on the stimulus-response relationship of the neuroactive steroids (Visser et al., 2002a). Dots represent the observed amplitudes. The thin lines represent the best-fitted stimulus-response relationship for all individuals. Intraindividual variability less 14% for all compounds.

which is consistent with our observations. Figure 8 also shows that especially for drugs with a log GABA-shift of around 0.1 to 0.2, a small change in the shape/location of the stimulus-response relationship may result in a large difference in the observed response. These observations might explain why it has been difficult to predict the intensity of the in vivo responses of benzodiazepines (Goggin et al., 2000). In this respect, it is important that the shape and the location of

the stimulus-response may differ between species, between individuals of the same species, and even within individual subjects. Such changes can occur as a result of differences/ changes in receptor expression.

In conclusion, a mechanism-based PK/PD model has been successfully applied to the effects of benzodiazepines, an imidazopyridine, a cyclopyrrolone, and a β-carboline in vivo. The model was able to separate the drug-receptor interaction
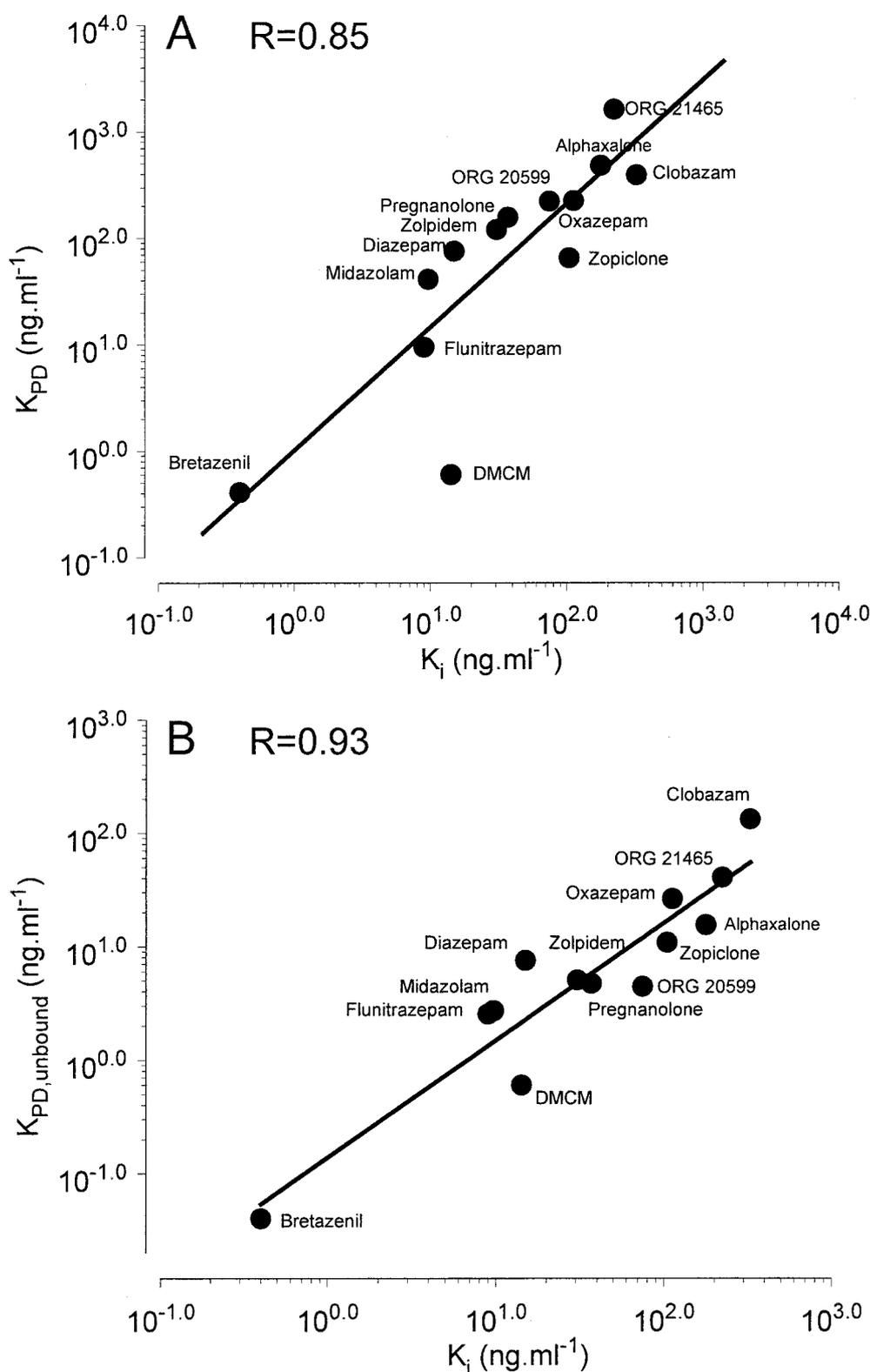
Fig. 6. In vitro-in vivo correlation of affinity estimates. On the x-axis are the K_i (nanograms per milliliter) values of benzodiazepines determined by displacing [^{3}H]flumazenil in presence of 50 μM GABA. On the y-axis the parameter estimates for K_{PD} (A) and the K_{PD} corrected for the protein binding (B, $K_{PD,unb}$) for benzodiazepines. The solid lines represent the relationship $\log(K_{PD}) = 1.15 \pm 0.22 \cdot \log(K_i) - 0.003 \pm 0.37$ ($r = 0.85$) (A) and $\log(K_{PD,unb}) = 1.03 \pm 0.12 \cdot \log(K_i) - 0.86 \pm 0.20$ ($r = 0.93$) (B). DMCM was not corrected for protein binding. The data for alphaxalone, pregnanolone, ORG 20599, and ORG 21465 were adapted from Visser et al. (2002a) and Anderson et al. (1997).

and the stimulus-response relationship. It was shown that all investigated compounds are low-efficacy modulators of the GABA$_A$ receptor, except for DMCM, which is a negative modulator. The stimulus-response relationship of the benzodiazepines was found similar to the first part of the stimulus-response relationship of neuroactive steroids and it was extended for the description of the response of a negative modulator, yielding a complex nonlinear stimulus-response relationship for the activation of the GABA$_A$ receptor in both positive and negative direction. Furthermore, the in vitro-in vivo correlations confirm that the new mechanism-based PK/PD model constitutes a realistic approach to the characterization of the effects of GABA$_A$ receptor modulators in vivo.
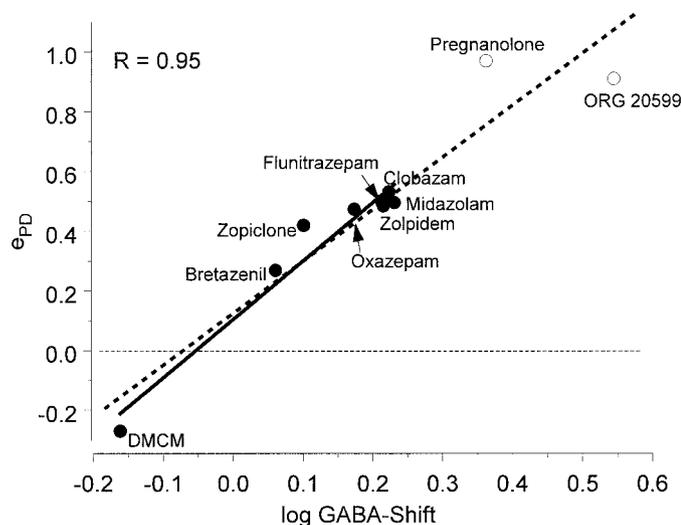
Fig. 7. In vitro-in vivo correlation of efficacy estimates. The logarithm of the GABA-shift is depicted on the *x*-axis. GABA-shift is defined as the ratio of the IC_{50} of displacing [^{3}H]flumazenil in presence and absence of 50 μM GABA. On the *y*-axis the parameter estimates for e_{PD} for benzodiazepines is depicted. The solid line represents the linear regression line: $e_{PD} = 1.96 \pm 0.19 \cdot \log(\text{GABA-shift}) + 0.11 \pm 0.03$ ($r = 0.97$) and the broken line represents the linear regression line: $e_{PD} = 1.75 \pm 0.21 \cdot \log(\text{GABA-shift}) + 0.14 \pm 0.05$ ($r = 0.95$) with GABA-shift for pregnanolone and ORG 20599 included, taken from McCauley et al. (1995) and Van Rijn et al. (1999), respectively.
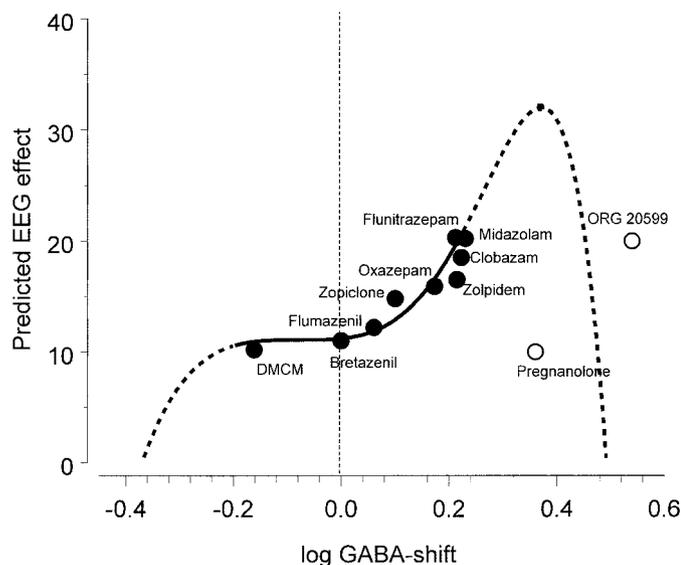

Fig. 8. Prediction of in vivo maximal EEG effects from in vitro GABA-shift. Substituting of the relationship between GABA-shift and e_{PD} obtained from graph 7, into eqs. 5 and 7, the in vivo maximal effects can be predicted from in vitro GABA-shift. Compounds with GABA-shifts lower than 1 are negative modulators and give negative EEG response compared with baseline. Compounds with GABA-shifts higher than 1 are positive modulators and give positive EEG response compared with baseline. It is predicted that compounds with GABA-shifts higher than 2 will give biphasic responses. The dots are the observed in vitro GABA-shifts and the in vivo maximal responses. Note that the GABA-shift is depicted in a logarithmic scale.

Acknowledgments

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