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Mechanism-Based Modeling of the Pharmacodynamic Interaction of Alphaxalone and Midazolam in Rats

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ABSTRACT

The objective of the present investigation was to characterize the pharmacodynamic interaction between the synthetic neuroactive steroid alphaxalone and the benzodiazepine midazolam. The time course of the electroencephalographic (EEG) effect (11.5–30 Hz) was determined in rats in conjunction with plasma concentrations. Alphaxalone was administered as a continuous intravenous infusion of 0, 1.2, 2.2, or 5.2 mg over 360 min. Midazolam was administered as a 5-min intravenous bolus infusion of 4 $mg \cdot kg^{-1}$. The pharmacokinetic profiles of both drugs were described by a two-compartment model. No pharmacokinetic interaction was observed. The EEG effect versus time profiles of midazolam and alphaxalone, when administered separately and in combination, were modeled on the basis of the recently proposed mechanism-based pharmacokinetic/pharmacodynamic model for $GABA_A$ receptor modulators, which contains separate expressions to describe the drug-receptor interaction and the stimulus-response relationship. The pharmacodynamic interaction between alphaxalone and midazolam was best characterized using an independent drug-drug interaction model without an expression for allosteric modulation of the effect of midazolam by alphaxalone. The final model contained an exponential expression to account for acute functional adaptation to the EEG effect upon continuous infusion of alphaxalone. The mechanism-based analysis showed that this functional adaptation is best explained by a change in the system-specific stimulus-response relationship, rather than the drug-receptor activation process. It is concluded that the pharmacodynamic interaction between alphaxalone and midazolam in vivo is best described using an independent interaction model without allosteric modulation.

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Recently, a mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) model was developed for the electroencephalographic (EEG) effects of a wide array of $GABA_A$ receptor modulators, including neuroactive steroids, benzodiazepines, imidazopyridines, cyclopyrrolones, and β-carbolines (Visser et al., 2002a,b, 2003a). A unique feature of this model is that it contains separate expressions for the characterization of 1) the receptor activation process and 2) the signal-transduction process. In this specific model, the receptor activation process is described by a hyperbolic function, whereas the biphasic transducer function is described by a parabolic equation (Fig. 1). It has been shown that a single unique transducer function can describe the effects of the different $GABA_A$ modulators. In this paradigm, neuroactive steroids and benzodiazepines differ in their ability to activate the receptor. It has also been shown that the model can account for the additive effects upon activation of two distinct $GABA_A$ receptor subtypes by zolpidem in vivo (Visser et al., 2003b).

The objective of the present investigation was to determine whether the mechanism-based PK/PD model could be used to characterize the effect of neuroactive steroids and benzodiazepines when administered in combination. This is of interest because this provides further information on the functioning of the $GABA_A$ receptor in vivo. Recently, mechanism-based PK/PD models have been proven successful for the quantification of drug-drug interactions (Tuk et al., 1999, 2002; Zuideveld et al., 2002). Specifically, these studies have focused on the competitive interaction between midazolam and α-hydroxy midazolam (Tuk et al., 1999), the allosteric interaction between midazolam and ethanol (Tuk et al., 2002), and the independent interaction between buspirone and its active metabolite (Zuideveld et al., 2002). The pharmacodynamic interaction between alphaxalone and midazolam is of interest, because there may be multiple mechanisms involved. A mechanism-based analysis of the pharmacodynamic interaction between the two may shed light on contri-

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ABBREVIATIONS: PK/PD, pharmacokinetic-pharmacodynamic; EEG, electroencephalogram; HPβCD, 2-hydroxy-propyl-β-cyclodextrin; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; AUE, area under effect curve; MVOF, minimum value of objective function.

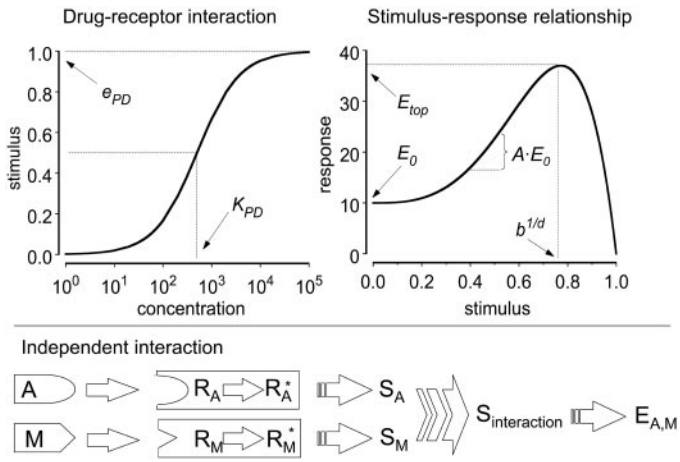

Fig. 1. Schematic view of the mechanism-based PK/PD model consisting of a model for drug-receptor interaction, which is a hyperbolic function of the effect-site concentration producing a stimulus. K_{PD} is the concentration producing the half-maximal stimulus and e_{PD} is the maximal stimulus. The second part consists of a biphasic stimulus-response model, which is represented by a parabolic function. 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. Below, a schematic view of independent interaction is shown. Two drugs bind to two receptors or receptor sites and produce both a stimulus. The combined stimulus is propagated into the response.

bution of each of these mechanisms to the interaction and thereby the functioning of this receptor in vivo. Furthermore, understanding of this interaction may provide information on the role of potent endogenous neuroactive steroids, which are derived from progesterone and deoxycorticosterone and might influence the effects of benzodiazepines (Majewska, 1992). Finally, it could be important for the optimization of the eventual treatment of e.g., epilepsy, anxiety, and sleep disorders with combinations of $GABA_A$ receptor modulators (Gasior et al., 1999; Czlonkowska et al., 2001).

Several studies have focused on the mechanisms of the interaction between neuroactive steroids and benzodiazepines. In vitro, it has been shown that neuroactive steroids can potentiate the effect of benzodiazepines (Harrison and Simmonds, 1984; Majewska, 1992; Gee et al., 1995). It has been demonstrated in vivo that neurosteroids can potentiate the anticonvulsant activity of diazepam (Gasior et al., 1997). Furthermore, synergy has been detected between pregnanolone and flurazepam in an EEG threshold model (Norberg et al., 1999). On the other hand, upon chronic treatment, neuroactive steroids have been shown to reduce the anticonvulsant activity of benzodiazepines (Czlonkowska et al., 2001).

The combined response to benzodiazepines and neuroactive steroids is mediated through specific and distinct binding sites (Paul and Purdy, 1992; Lambert et al., 1995). When two drugs produce the same pharmacological effect via different receptor sites, their effector pathways converge somewhere in the sequence of events between receptor activation and effect (Fig. 1). In the case of the pharmacodynamic interaction between alphaxalone and midazolam, however, several specific mechanisms need to be taken into consideration. First, it can be hypothesized that the two drugs, which bind to distinct binding sites, compete for the same intermediate (stimulus) in a shared pathway, resulting in an independent interaction. In addition, it can be expected that when allosteric modulation is prominent, this might be reflected in a decrease of the in vivo affinity. A final factor, which needs to be taken into consideration, is the possible development of functional adaptation as a result of receptor desensitization and/or an altered transducer function.

In the present investigation, based on the observations and simulations, a mechanism-based PK/PD model was proposed for the pharmacodynamic interaction that contained a specific expression for the functional adaptation to the EEG effect.

Materials and Methods

Animals and Surgical Procedures. The protocol of this investigation was approved by the Ethical Committee on Animal Experimentation of Leiden University. The studies were conducted in accordance with the requirements of national legislation and appropriate guidelines for animal care. Male Wistar rats (309 ± 33 g, n = 59; Charles River BV, Zeist, The Netherlands) were used throughout the study. After surgery, the animals were housed in standard plastic cages with a normal 12-h day/night schedule with lights on at 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. During the experiments, the animals were deprived of food and water.

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., 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, in the right jugular vein for the 5 min midazolam infusion and in the right femoral vein for the 360-min alphaxalone infusions. The cannulae, filled with heparinized 25% polyvinylpyrrolidone 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. of medetomidine hydrochloride (Domitor; Pfizer, Capelle a/d IJssel, The Netherlands) and 1 mg · kg^{-1} s.c. of ketamine base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands). After the surgery, 4 mg of ampicillin (A.U.V., Cuijk, The Netherlands) was administered to aid recovery.

Drugs and Dosages. Alphaxalone (5β-pregnan-3β-ol-11,20-dione; ICN Biochemicals BV, Asse-Relegem, Belgium) was dissolved in a vehicle of 25% (w/v) HPβCD (hydroxypropyl-β-cyclodextrin; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) in saline at concentrations of 0.61 mg · ml^{-1}, 1.22 mg · ml^{-1}, and 2.89 mg · ml^{-1}, respectively. A solution of 12.5 mg · ml^{-1} midazolam (Duchefa Pharma BV, Haarlem, The Netherlands) in dimethyl sulfoxide (DMSO) was prepared. The rate of the continuous infusion of alphaxalone was 5 μl · min^{-1} and the 5-min infusion of midazolam or vehicle was administered at a rate of 100 μl · min^{-1}. The rats were randomly assigned to four treatment groups of 12 to 17 rats that received a 360-min (or 425-min for group 2) continuous infusion of alphaxalone or the vehicle (25% HPβCD). After 60 min, a 5-min infusion of midazolam was administered to the subgroups 1A, 2A, 3A, and 4A and a 5-min infusion of the vehicle (DMSO) to subgroups 1B, 2B, 3B, and 4B. An overview of the various treatments is given in Table 1.

In Vivo Interaction Experiments. All experiments were started between 8:30 AM and 9:30 AM to exclude the influence of circadian rhythms. The rats were placed in a rotating drum to control the level of vigilance, thereby avoiding the interference of sleep patterns. Bipolar EEG leads (F_l-C_l) were continuously recorded

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TABLE 1

Overview of the alphaxalone and midazolam treatment for groups 1 to 4

The groups 1 to 4 received a 360- or 425-min continuous infusion of alphaxalone or vehicle

In addition, the groups 1A, 2A, 3A, and 4A received a 5-min infusion of 1.25 mg of midazolam (4.0 ± 0.3 mg · kg^{-1}) and the groups 1B, 2B, 3B, and 4B received a 5-min infusion of the vehicle. Depicted are dose, rate, and duration of the continuous infusion, the 5-min infusion, the number of animals, and averaged body weight. Vehicle was 25% (w/v) HPβCD for alphaxalone and 100 μl of DMSO for midazolam.

	Continuous Infusion of Alphaxalone or Vehicle			5-min Infusion of Midazolam or Vehicle	Number of Animals	Weight
	Dose	Rate	Duration	Group A/Group B	Group A/Group B	Group A/Group B
	mg	$\mu g \cdot min^{-1}$	*min*	*mg*		*g*
1A/1B	Vehicle	Vehicle	360	1.25/vehicle	8/4	318 ± 8/323 ± 19
2A/2B	1.20	3.06	425	1.25/vehicle	9/8	298 ± 12/286 ± 9
3A/3B	2.20	6.10	360	1.25/vehicle	7/7	313 ± 13/307 ± 12
4A/4B	5.20	14.4	360	1.25/vehicle	9/7	319 ± 8/329 ± 14

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 digitized signal was fed into a Pentium III computer and stored on hard disk for off-line analysis. The EEG was recorded continuously for 470 min. At t = 45 min, a 360-min (or 425-min) zero-order intravenous infusion of alphaxalone was started and at t = 105 min (165 min for some individuals in group 1A and 2A), a 5-min zero-order intravenous infusion of midazolam was administered to the conscious rats using an infusion pump (BAS Bioanalytical Systems, West Lafayette, IN). Quantitative EEG parameters were obtained off-line for each 5-s epochs by Fast Fourier Transformation with a user-defined script within the data analysis software package Spike 2 for windows, version 3.18 (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 to 2.0 ml during each experiment. In the groups 2B, 3B, and 4B, 20 samples were taken for determination of alphaxalone pharmacokinetics. In the groups 1A, 2A, 3A, and 4A, six alphaxalone and 12 midazolam samples were taken, respectively. The blood samples were immediately heparinized and centrifuged at 5000 rpm for 15 min for plasma collection and were stored at −20°C until HPLC analysis (total number of samples per rat).

HPLC Analysis. The plasma concentrations of alphaxalone were determined by HPLC with fluorescence detection as described previously (Visser et al., 2000). Linear calibration curves were obtained in the range 0.025 to 10 $\mu g \cdot ml^{-1}$ and the limit of quantification was 0.025 $\mu g \cdot ml^{-1}$. The inter- and intra-assay variability were 30 and 15% for 0.5 and 5 $\mu g \cdot ml^{-1}$, respectively.

The plasma concentrations of midazolam were determined by HPLC and UV detection as described previously (Visser et al., 2003a). Linear calibration curves were obtained in the range 0.01 to 10 $\mu g \cdot ml^{-1}$. Inter- and intraday variability and the extraction recovery were determined using two quality controls (0.25 and 2.5 $\mu g \cdot ml^{-1}$). The limit of quantification, inter- and intra-assay variability, and extraction recovery of midazolam were 0.025 $\mu g \cdot ml^{-1}$, and 11, 6, and 110%, respectively.

Pharmacokinetic Data Analysis. Compartmental pharmacokinetic analysis was performed by fitting a standard two-compartment model to the concentration-time profiles of the compounds by use of the ADVAN3 TRANS4 subroutine for midazolam (Visser et al., 2003a) and the ADVAN6 subroutine for alphaxalone (Visser et al., 2002a) within the nonlinear mixed effect modeling software package NONMEM (version V; 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 as well as intraindividual residual error. The pharmacokinetic parameters: clearance (Cl) and the intercompartmental clearance (Q) were modeled as function of body weight (BW) as described previously (Visser et al., 2002a):

$$Cl_i = \theta_i \cdot BW_i^{\theta_j} \quad (1)$$

$$Q_i = \theta_i \cdot BW_i^{\theta_j} \quad (2)$$

where θ_j was fixed at the previously obtained value (1.67, Visser et al., 2002a).

The interindividual variability of these parameters was modeled according to an exponential equation.

$$P_i = \theta_i \cdot \exp(\eta_i) \quad (3)$$

where θ_i 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 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:

$$C_{mij} = C_{P_{ij}} \cdot (1 + \varepsilon_{ij}) \quad (4)$$

where C_{pij} represents the jth plasma concentration for the ith individual predicted by the model. C_{mij} represents the measured concentration, and ϵ_{ij} accounts for the residual deviation of the model-predicted value from the observed concentration. The value for ϵ was assumed independently normally distributed with mean zero and variance σ^2. The first-order conditional estimation method with interaction (FOCE interaction) was used to estimate the population θ, ω^2, and σ^2. From individual Bayesian post hoc parameter estimates, Cl, Q, V_1, V_2, volume of distribution at steady state (V_{dss}), and half-life were calculated following standard procedures (Gibaldi and Perrier, 1982). Individual parameter estimates were used to calculate individual alphaxalone and midazolam concentrations at the time points of the EEG measurements.

Pharmacodynamic Analysis of the Continuous Infusions of Alphaxalone. The concentration-effect relationship of alphaxalone was analyzed according to the recently proposed mechanism-based PK/PD model for $GABA_A$ receptor modulators, which features separate expressions for the characterization of the receptor activation process and the transducer function (Visser et al., 2002a,b, 2003a). In the mechanism-based model, the response is considered a function of the stimulus induced by the drug-receptor binding (Fig. 1). Upon binding to the receptor, the drug produces a stimulus, which is followed by a cascade of 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 receptor activation can be different for different drugs. The stimulus-response relationship, on the other hand, is the same, regardless of the drug tested. In this model,

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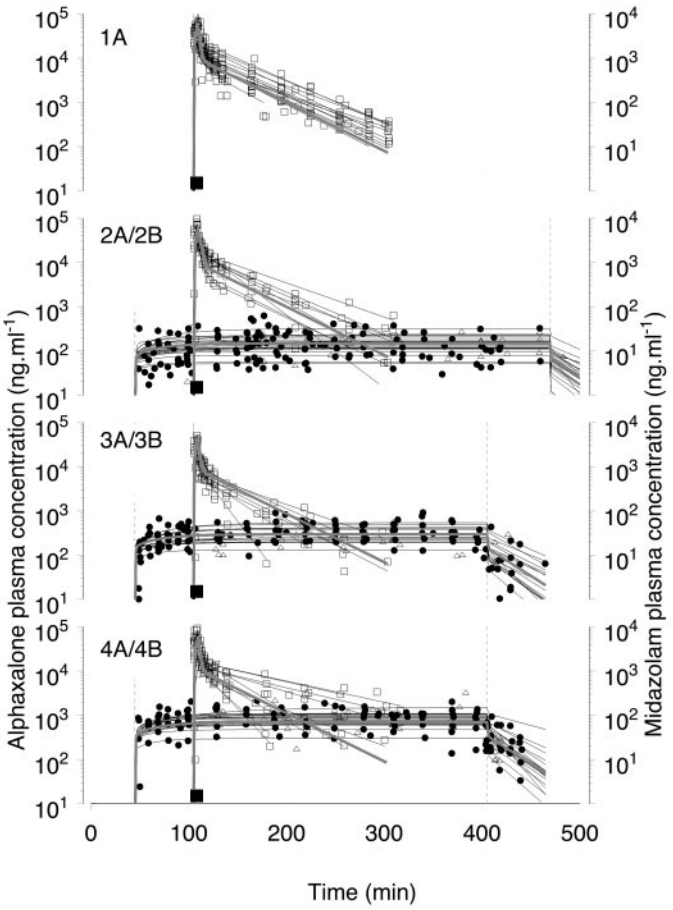

Fig. 2. Observed alphaxalone (closed triangles and circles) and midazolam (open squares) plasma concentration-time profiles for the treatment groups 1A/B, 2A/B, 3A/B, and 4A/B. The individual predictions (thin black lines) and population predictions (thick gray lines) are depicted. The triangles correspond to the alphaxalone concentrations in the A groups and the circles to the B groups. The *x*-axis represents the time in minutes, the plasma concentration of alphaxalone (nanograms per milliliter) is depicted on the left *y*-axis and the plasma concentration of midazolam (nanograms per milliliter) on the right *y*-axis both on a logarithmic scale. The black boxes represent the duration of the midazolam infusion. The dotted reference lines indicate the start and stop time of the alphaxalone/vehicle infusion.

the interaction of the drug with the receptor yields a stimulus S according to the following equation.

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

where C represents the concentration of the drug, K_{PD} the in vivo estimated affinity, and e_{PD} is the in vivo estimated efficacy, relative to 1. As was shown previously, alphaxalone reached the maximal observed stimulus upon short i.v. infusion, which was set to 1 (Visser et al., 2002a). This stimulus is propagated into the ultimate effect (E) for which the relation to the stimulus is given by a function f.

$$E = f(S). \quad (6)$$

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

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

where E_{top} represents the top of the parabola, a is a constant determining the height 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 results in an asymmetry of the parabola. When no drug is present the EEG effect is equal to its baseline value (E_0) and eq. 7 reduces to the following.

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

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

$$a = A \cdot E_0, \quad (9)$$

in which A is a linear proportionality constant. Substituting eqs. 8 and 9 into eq. 7, and rearrangement yields the following.

$$E = E_0 \cdot (1 - A \cdot ((S^d)^2 - 2 \cdot b \cdot S^d)). \quad (10)$$

In the present mechanism-based PK/PD analysis of the combined effect of alphaxalone and midazolam, it was first investigated whether the in vivo potency, in vivo efficacy and/or stimulus-response relationship of alphaxalone were altered under steady-state conditions (i.e., a continuous i.v. infusion over 360 min) compared with results obtained upon short (5-min i.v.) infusions (Visser et al., 2002a). For this purpose, the data about the concentration-effect relationship of alphaxalone of the groups 1B, 2B, 3B, and 4B and the parts of the relationship from groups 2A, 3A, and 4A (before the midazolam infusion, i.e., until t = 105 min) were extracted. The concentration-effect relationships were modeled using the mechanism-based PK/PD model. To account for tolerance development upon the continuous infusion of alphaxalone, the parameters K_{PD}, e_{PD}, or A were allowed to increase or decrease in time during the continuous infusion of alphaxalone. The increase in K_{PD} and the decrease in e_{PD} and A with time were defined as,

$$K_{PD(t)} = K_{PD} \cdot e^{D_1 \cdot t} \quad (11)$$

$$e_{PD(t)} = e_{PD} \cdot e^{-D_2 \cdot t} \quad (12)$$

$$A_{(t)} = A \cdot e^{-D_3 \cdot t} \quad (13)$$

TABLE 2

Population pharmacokinetic parameter estimates for Cl, Q, V_1, and V_2 with the corresponding interindividual coefficient of variation (CV%) and 95% confidence interval (CI) for alphaxalone

Intraindividual residual variation was 41% for alphaxalone.

	n	Cl	Q	V_1	V_2
		$ml \cdot min^{-1} \cdot kg^{-1}$	$ml \cdot min^{-1} \cdot kg^{-1}$	$ml \cdot kg^{-1}$	$l \cdot kg^{-1}$
Alphaxalone		150 ± 9	167 ± 39	96 ± 11	1120 ± 196
95 % C.I.		132–168	89–245	74–118	728–1512
CV%		(41%)	(67%)	(<1%)	(63%)
2A/B	17	186 ± 17	192 ± 13	97 ± 0.2	1391 ± 110
3A/B	14	165 ± 12	189 ± 11	96 ± 0.1	1248 ± 121
4A/B	16	127 ± 10	161 ± 12	96 ± 0.1	950 ± 91

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TABLE 3
Population pharmacokinetic parameter estimates for Cl, Q, V_1 and V_2 with the corresponding inter-individual coefficient of variation (C.V.%) and 95% confidence interval (C.I.) for midazolam. Intra-individual residual variation was 34% for midazolam

	n	Cl	Q	V_1	V_2
		$ml \cdot min^{-1} \cdot kg^{-1}$	$ml \cdot min^{-1} \cdot kg^{-1}$	$ml \cdot kg^{-1}$	$l \cdot kg^{-1}$
Midazolam		58.8 ± 4.3	88.7 ± 7.0	207 ± 33	1330 ± 69
95 % CI		51–67	75–103	141–273	1192–1468
CV %		(45%)	(16%)	(95%)	(27%)
1A	8	71 ± 9	89 ± 2	282 ± 109	1395 ± 110
2A	9	58 ± 6	88 ± 2	295 ± 82	1331 ± 92
3A	7	95 ± 10[a]	90 ± 2	518 ± 263	1457 ± 122
4A	9	53 ± 8	88 ± 2	333 ± 61	1245 ± 79

[a] Significantly higher than group 2A and 4A, $p < 0.05$.

respectively, where D_1 represents the rate constant for the increase of K_{PD} with time and D_2 and D_3 represent the rate constant for the decrease of e_{PD} and A with time, respectively. The parameters D_1, D_2, and D_3 were fixed at 0 when K_{PD}, e_{PD}, and A were not allowed to change with time. Furthermore, the parameters characterizing the shape of the stimulus-response relationship were fixed at the previously obtained values with the corresponding interindividual variability [A = 9.2 (22%), b = 0.44 (7%), and d = 3.36 (−), respectively; Visser et al., 2002a,b].

Mechanism-Based Modeling of the Pharmacodynamic Interaction. Neuroactive steroids (alphaxalone) and benzodiazepines (midazolam) are known to bind to distinct binding sites at the $GABA_A$ receptor and it has been shown that these $GABA_A$ receptor modulators differ with respect to their affinity and efficacy but share the same stimulus-response relationship (Visser et al., 2002a,b, 2003a). As shown in a previous investigation, drug activation of different binding sites can each result in EEG effects, which are in principle additive (Visser et al., 2003b). In principle, however, the total stimulus cannot exceed the maximum value of 1. For example, consider that two drugs have affinity for different sites on a nuclear receptor. Suppose further that occupation of these sites by A and B independently activates the receptor. If a fraction x_A of the available receptors is activated by A, maximally a fraction of $(1 - x_A)$ remains

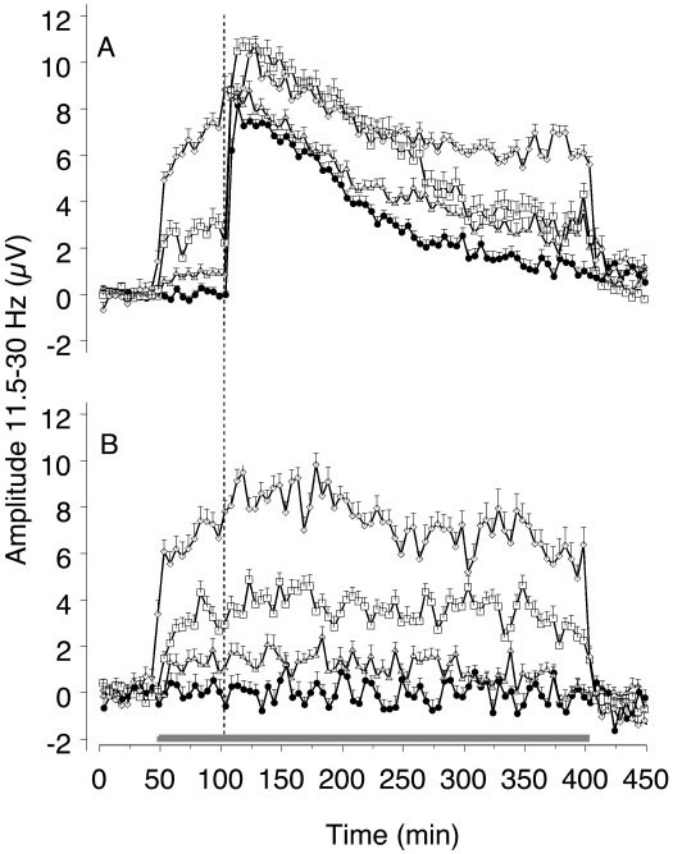

Fig. 3. Averaged (mean ± S.E.) time-effect profiles for all treatment groups. A, effects of midazolam with increasing concentrations alphaxalone for treatment groups 1A (circles), 2A (squares), 3A (triangles), and 4A (diamonds). B, effects of alphaxalone with increasing concentrations for treatment groups 1B (circles), 2B (squares), 3B (triangles), and 4B (diamonds). Before averaging of 5-min time intervals, individual baseline values were subtracted from the individual time-effect profiles. The gray bar represents the duration of the continuous infusion of the vehicle HPβCD or alphaxalone and the dotted vertical line represent the start of the 5-min midazolam infusion. On the x-axis, the time in minutes is depicted and on the y-axis, the EEG is depicted as amplitude in the 11.5- to 30-Hz band relative to the baseline.

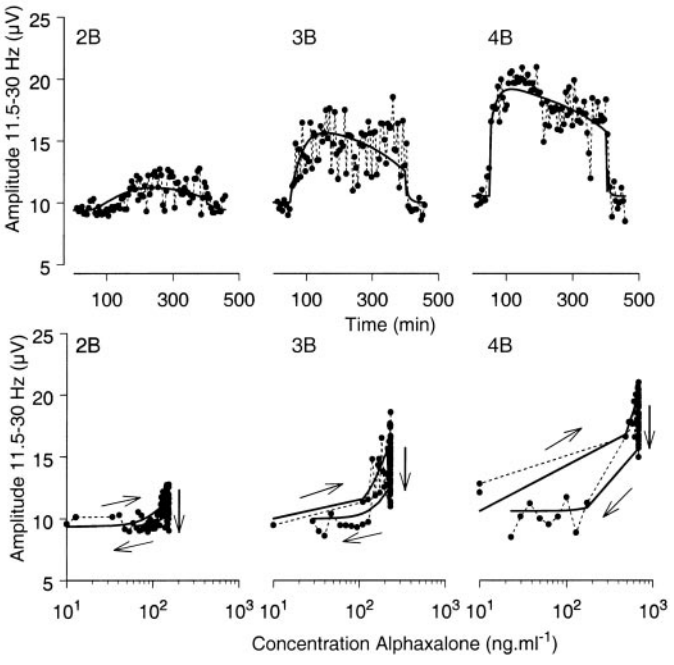

Fig. 4. Top, individual (dots) and predicted (lines) effect-time profiles of alphaxalone for selected representative rats in treatment groups 2B, 3B, and 4B. At t = 45 min, the continuous infusion of alphaxalone was started with the infusion rates 3.06, 6.0, and 14.4 μg/min, respectively. On the y-axis, the EEG is depicted as amplitude in the 11.5- to 30-Hz band and on the x-axis the time in minutes. The predicted effect-time profiles are shown for the case that the A was allowed to change in time (Table 4). Bottom, observed and predicted concentration-effect relationships for the selected representative rats in treatment groups 2B, 3B, and 4B. The arrows indicate the direction of the proteresis in the concentration-effect relationships.

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available for activation by B. The model is coined an "independence" model because it is based on a concept of noninteraction, first proposed by Bliss (1939). This independent drug interaction model (Fig. 1) was incorporated into eq. 10

$$\frac{S_{\text{interaction}}}{S_{\max}} = \frac{S_{\text{alph}}}{S_{\max}} + \frac{S_{\text{mida}}}{S_{\max}} - \frac{S_{\text{alph}} \cdot S_{\text{mida}}}{S_{\max}^2} \quad (14)$$

where $S_{\text{interaction}}$ is the combined stimulus of both drugs and S_{alph} and S_{mida} are the drug stimuli of alphaxalone and midazolam alone, respectively. $S_{\max}$ is the maximal achievable stimulus of the system. Because the maximum achievable stimulus in the system ($S_{\max}$) equals 1, substituting eq. 5 for both drugs into eq. 14 yields the following.

$$\frac{S_{\text{interaction}}}{S_{\max}} = \frac{e_{\text{PDA}} \cdot C_{\text{A}}}{C_{\text{A}} + K_{\text{PDA}}} + \frac{e_{\text{PDM}} \cdot C_{\text{M}}}{C_{\text{M}} + K_{\text{PDM}}} - \frac{e_{\text{PDA}} \cdot C_{\text{A}}}{C_{\text{A}} + K_{\text{PDA}}} \cdot \frac{e_{\text{PDM}} \cdot C_{\text{M}}}{C_{\text{M}} + K_{\text{PDM}}} \quad (15)$$

where K_{PDA} and e_{PDA} represent the in vivo affinity and efficacy of alphaxalone and K_{PDM} and e_{PDM} represent the in vivo affinity and efficacy of midazolam, respectively. To account for the functional adaptation upon the continuous infusion of alphaxalone, the parameters K_{PDA}, e_{PDA}, or A were allowed to change with time upon infusion of alphaxalone according to the eqs. 11 to 13.

In the modeling procedure, the parameters determining the interaction between midazolam and alphaxalone at the stimulus level (eq. 15 were estimated, whereas the parameters determining the shape of the stimulus-response relationship were fixed at the values described in the previous paragraph. Interindividual variability for the parameter K_{PD} was modeled using an exponential error model (eq. 3) and for e_{PD} and E_0 using a proportional error model as follows.

$$P_{\text{i}} = \theta_1 \cdot (1 + \eta_i) \quad (16)$$

The residual variability in the pharmacodynamics was modeled as a CCV error according to eq. 4. The FOCE interaction method 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.

Simulations for Figs. 6, 8, 9, and 10 were performed using the software package Berkeley Madonna 8.0 (Macey and Oster, University of California at Berkeley, Berkeley, CA).

Statistical Analysis. Goodness-of-fit was analyzed on the basis of visual inspection and the value of the objective function. Model selection was based on the Akaike information criterion (Akaike, 1974) and assessment of parameter correlation. 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 (GraphPad Software Inc., San Diego, CA). All data are represented as mean ± S.E. and $P < 0.05$ was considered significant.

Results

Pharmacokinetics. Figure 2 shows the observed, predicted population, and individual alphaxalone and midazolam plasma concentration-time profiles for all treatment groups. The pharmacokinetic profiles of alphaxalone and midazolam were best described by a two-compartment pharmacokinetic model. Population estimates and averaged Bayesian post hoc estimates parameter estimates are summarized in Table 2 for alphaxalone and in Table 3 for midazolam.

For alphaxalone, fixing of the exponent on the body weight improved the fits, because individual weight ranged between 255 and 397 g. The population estimates of alphaxalone for Cl and Q were estimated at $150 \cdot \text{BW}^{1.67}$ and $167 \cdot \text{BW}^{1.67}$ ml · min^{-1} · kg^{-1}, respectively. Alphaxalone showed a distribution half-life of 0.6 ± 0.02 min and an elimination half-life of 22.4 ± 0.8 min (n = 47). No significant differences were observed in the pharmacokinetic parameter estimates between the treatment groups of alphaxalone. Furthermore, the post hoc parameter estimates of groups A versus B showed no significant differences, indicating that midazolam did not have an influence on the pharmacokinetics of alphaxalone. In addition it was observed that alphaxalone reached steady-state concentrations in all treatment groups before midazolam was administered. The alphaxalone dosages were chosen based on the desired target steady-state levels of 100, 300, and 700 ng · ml^{-1} alphaxalone. The observed steady-state levels were 144 ± 12, 299 ± 30, and 813 ± 65 ng · ml^{-1} in treatment groups 2, 3, and 4, respectively.

For midazolam a distribution half-life of 0.9 ± 0.4 min and an elimination half-life of 27.8 ± 3.0 min was found (n = 33). Estimates for clearance for group 3A were significantly higher than estimates for group 2A and 4A. The pharmacokinetic parameter estimates were investigated for the level of alphaxalone as covariate, but no relationship was found between the level of alphaxalone and pharmacokinetic parameter estimates, i.e., alphaxalone did not influence the pharmacokinetics of midazolam and vice versa.

Pharmacodynamic Interaction between Midazolam and Alphaxalone. From the individual effect-time profiles, the individual baseline values were subtracted and subsequently, the observed effects were averaged over 5-min time intervals. The averaged effect-time profiles, expressed as amplitude in 11.5- to 30-Hz frequency band, of all treatment groups are shown in Fig. 3. No influence on the effect parameter was observed for the treatment with a continuous infusion of the vehicle HPβCD and a 5-min infusion of DMSO (group 1B, bottom). Upon infusion of alphaxalone, a concentration-dependent increase of the EEG effect parameter was observed. No differences in this effect were observed between the treatment groups A and B. Upon infusion of midazolam, the EEG effect immediately increased. This pattern was similar for the different treatment groups, except for the treatment group 4A, where the absolute effect was much smaller and followed by a small decrease before the maximal effect was reached at 25 min after infusion of midazolam. Although this minor effect is not clearly visibly as a result of the averaging over 5-min intervals, it was clearly observed for six of nine rats in group 4A (see representative profile in Fig. 6). The effect of midazolam reached a height of 8, 9, 11, and 11 μV relative to baseline (in the absence of alphaxalone) in groups 1A, 2A, 3A, and 4A, respectively. To compare the

TABLE 4
Population parameter estimates (θ ± S.E.) for the drift in time of the parameters K_{PD}, e_{PD} and A and the corresponding MVOF for the concentration-effect data upon administration of alphaxalone alone
Intraindividual residual variation was 11%.

Parameter	Change in Time	Estimate ± S.E. min^{-1}	MVOF
None			5854
K_{PD}	D_1	$1.5 \cdot 10^{-3} \pm 3.2 \cdot 10^{-4}$	5322[a]
e_{PD}	D_2	$7.1 \cdot 10^{-4} \pm 2.9 \cdot 10^{-6}$	5353[a]
A	D_3	$1.6 \cdot 10^{-3} \pm 0.3 \cdot 10^{-4}$	5419[a]

[a] Significantly lower compared with no change in time of a parameter, $p < 0.05$.

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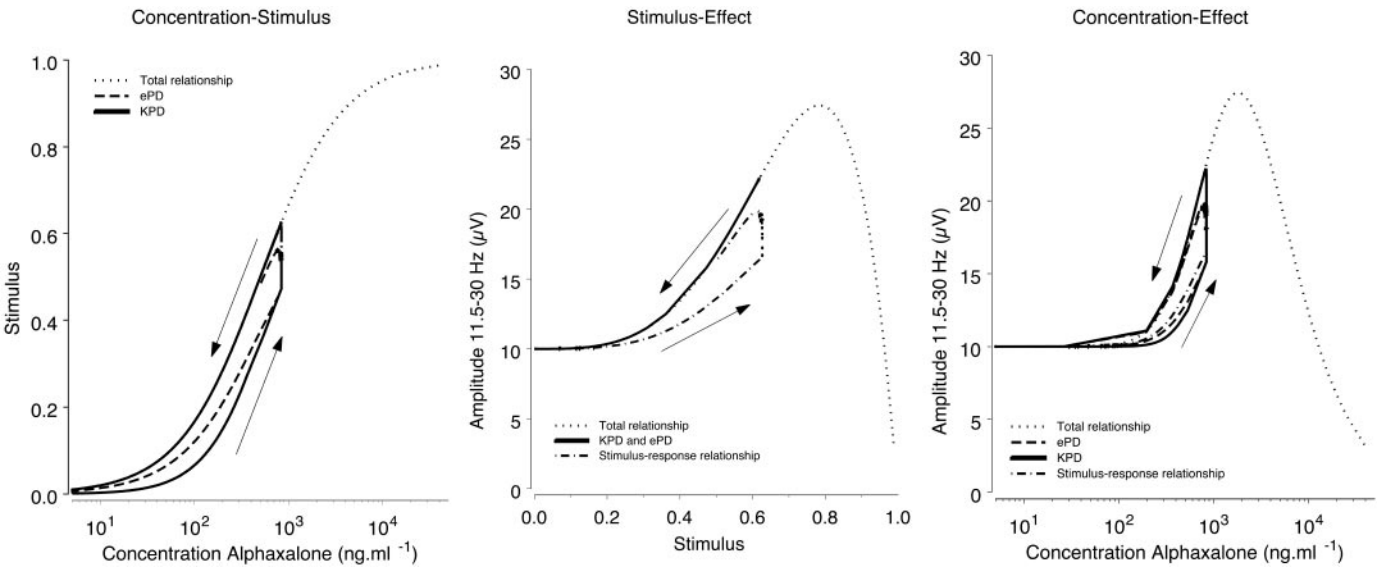

Fig. 5. Simulations of the concentration-stimulus relationship, the stimulus-effect relationship and the resulting concentration-effect relationship based on functional adaptation due to parameter e_{PD}, K_{PD}, and A. In the first panel, the concentration-stimulus relationship is shown. This relationship is altered when efficacy (dotted line) or affinity (solid line) is decreased, which is superimposed on the total relationship without tolerance. In the second panel, the stimulus-response relationship is shown, which only altered compared with the total relationship when adaptation occurs on the stimulus-response level. In the last panel, the concentration-effect profiles are simulated with the 5.2 mg of alphaxalone over 360 min. A functional adaptation occurring in e_{PD}, K_{PD}, or A does result in similar concentration-effect relationships. The arrows indicate the direction of the proteresis.

effects of increasing dosages of alphaxalone and the effect of midazolam under influence of increasing dosages of alphaxalone, the effect-time profiles in Fig. 3 are depicted again in Fig. 4 without the standard errors. A concentration-dependent increase in the EEG effect was observed during the continuous infusion of alphaxalone (Fig. 4, bottom). However, the EEG effect decreased in time despite the constant plasma concentrations. This observation indicates development of functional adaptation due to prolonged exposure to alphaxalone, because the pharmacokinetic profiles showed constant concentrations over time.

In the treatment groups 1A and 2A, the increase in the EEG effect due to administration of midazolam was quantitatively similar (area under the effect curve (AUE values), calculated relative to the baseline of alphaxalone treatment for mean effect curves, were 1136 and 1131 $\mu V \cdot min$, respectively). The midazolam effects in groups 3A and 4A were slightly (AUE value 808 $\mu V \cdot min$) and significantly (98 $\mu V \cdot min$) reduced compared with the effect of the control group 1A, respectively.

Pharmacodynamic Analysis of the Effect upon Continuous Infusions of Alphaxalone. Figure 4 shows the results of the fitting of the mechanism-based model to the effect versus time profiles of alphaxalone during continuous infusion. In the concentration-effect relationship of alphaxalone proteresis was observed, indicating the development of functional adaptation as shown in Fig. 4, bottom. The concentration-effect relationships were modeled using the mechanism-based PK/PD model, allowing K_{PD}, e_{PD}, or A to increase or decrease in time according to a first-order process. The parameters D_1, D_2, and D_3 were fixed at zero for the cases they were not allowed to change in time. The modeling results are shown in Table 4. It was found that a change in time of parameter A, K_{PD}, or e_{PD} significantly improved the goodness of fit as reflected in a reduction of the minimum value of objective function (MVOF). The increase of the parameter K_{PD} in time resulted in the lowest MVOF. Although a time-dependent increase in K_{PD} yielded a significantly lower MVOF, the fits obtained by a decrease in e_{PD} and A were not visually different from the fit with a time-dependent increase in K_{PD}. The observed and the predicted effect-time courses upon a change in A in time are shown for three representative individuals from group 2B, 3B, and 4B in Fig. 4, top. The observed and predicted concentration-effect profiles of the same individuals are shown in Fig. 4, bottom. The arrows indicate the time direction of the concentrations. Via simulations with the estimated parameters, it could be shown that the observed tolerance due to an altered drug-receptor interaction (i.e., change K_{PD} or e_{PD}) or an altered stimulus-response relationship (i.e., change in A) could not be distinguished in the concentration range of alphaxalone studied in this investigation. The results of these simulations are shown in Fig. 5. Based upon inspection of post hoc estimates, simulations, and modeling of the total data set, including midazolam, as explained in the following paragraph, it was ultimately found that a decrease in the stimulus-response relationship best described the functional adaptation.

Mechanism-Based PD Analysis of the Interaction between Alphaxalone and Midazolam. The mechanism-based PK/PD model in combination with the independent drug-drug interaction model was fitted to the concentration-effect data for all individuals. The final modeling runs were performed with the data of the treatment groups A averaged over 1-min time intervals and the data of treatment groups B averaged over 5-min time intervals. In this analysis, the parameter estimates obtained for the treatment groups B were not different from the estimates obtained when fitting the data from treatment groups B averaged over 1-min time intervals in the paragraph above.

In the modeling procedure, it was investigated whether the tolerance was related to a decrease in the stimulus response

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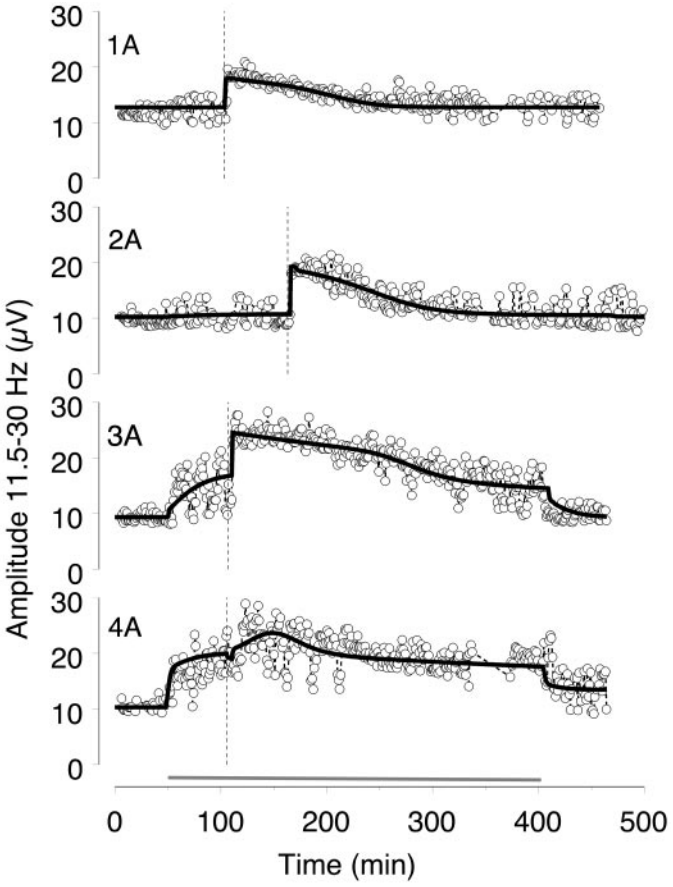

Fig. 6. Selected representative individual observed (dots) and predicted (lines) effect-time profiles of the interaction of midazolam with increasing levels of alphaxalone (groups 1A, 2A, 3A, and 4A). At t = 50 min, the continuous infusion (gray bar) was started of the vehicle HPβCD (group 1A/B) or the infusion rates (3.06, 6.0, and 14.4 μg/min) of alphaxalone (groups 2A/B, 3A/B, and 4A/B). At t = 105 or 165 (dotted lines) 1.25 mg of midazolam was administered in a 5-min infusion. On the y-axis, the EEG is depicted as amplitude in the 11.5- to 30-Hz band and on the x-axis the time in minutes. The predicted effect-time profiles were obtained when allowing the stimulus-response relationship to decrease in time.

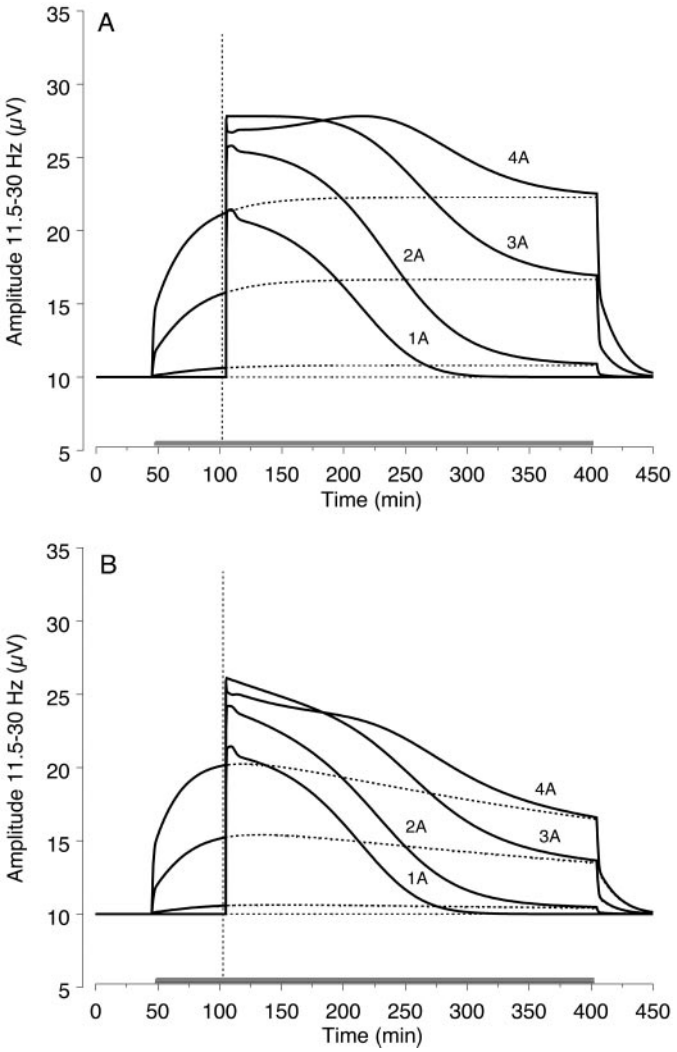

Fig. 7. Simulations of the effect profiles of all treatment groups in the absence (A) or presence (B) of functional adaptation in the stimulus-response relationship. It is predicted that the combined effect in group 4A reaches E_{top} and therefore shows a biphasic effect profile. However, due to functional adaptation, E_{top} is reduced (B), which is also observed in the experiments. Solid lines represent the treatment groups A and the dotted lines the treatment groups B, respectively.

relationship, a decrease in e_{PD} or an increase in K_{PD}. In this analysis, it could be shown that a decrease in the stimulus-response relationship described the observed adaptation best, based on the observations for the effects of midazolam in the highest dosing group. The observed and predicted effect-time course of four representative rats from treatment groups 1A, 2A, 3A, and 4A are shown in Fig. 6. The effects of midazolam in group 1A, 2A, and 3A were only different with respect to the initial elevation by alphaxalone, whereas for the individual from group 4A, a small decrease in effect intensity was observed before the maximal effect was reached. This observation could only be described and explained by a decrease in the stimulus-response relationship.

In Fig. 7, simulations are shown of the effect profiles in the absence (A) or presence (B) of functional adaptation in the stimulus-response relationship. In simulations with an altered K_{PD} or e_{PD}, the maximal observed effect of the interaction can be reduced, but then the typical profile of group 4A cannot be predicted (simulations not shown). Because this effect, observed for group 4A, took place in less than 10% of the total time profile and only in six individuals, this did not affect the MVOF. The parameter estimates are shown in Table 5. Statistical analysis showed that there were no differences between the post hoc parameter estimates of the treatment groups. The height of the stimulus-response relationship decreased in time (D_3 = 0.0018 ± 0.0001 min^{-1}) via parameter A. With the population parameter estimates, the decrease in stimulus-response relationship was simulated to show the behavior during the experiment compared with the stimulus-response relationship upon bolus dosing, which is shown in Fig. 8A. As comparison, in Fig. 8B, the observed stimulus-response relationship is shown. The individual predictions for the stimulus-response relationship are omitted for clarity.

In the present investigation, no data were available to characterize the full stimulus-response relationship (i.e., reaching a maximum stimulus of 1). However, including the alphaxalone concentration-effect data obtained upon 5-min intravenous infusion from a previous investigation (Visser et al., 2002a) showed that the development of functional adaptation did not influence the time course of the alphaxalone

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TABLE 5

Population pharmacodynamic parameter estimates for the interaction between alphaxalone and midazolam: E_0, e_{PDA}, K_{PDA}, e_{PDM}, and K_{PDM}, ($\theta \pm$ S.E.) with the corresponding interindividual coefficient of variation (CV%) and 95% confidence interval (CI) for all groups that received 1.25 mg of midazolam in 5-min infusion

The decrease in the height of the stimulus-response relationship was 0.0018 ± 0.0001 min^{-1}. Intraindividual residual variation was for each group lower than 12%.

Group	E_0	e_{PDA}	K_{PDA}	e_{PDM}	K_{PDM}
	μV		$ng \cdot ml^{-1}$		$ng \cdot ml^{-1}$
Population	11.9 ± 0.25	1 fixed	553 ± 33	0.68 ± 0.02	28 ± 19
95% CI	11.4–12.4		487–619	0.64–0.72	0–66
CV%	(40%)	(−)	(77%)	(43%)	(236%)
1A	10.0 ± 0.6	n.a.	n.a.	0.54 ± 0.04	17 ± 8
1B	11.8 ± 0.7	n.a.	n.a.	n.a.	n.a.
2A	9.1 ± 0.3	1 fixed	520 ± 215	0.61 ± 0.06	72 ± 63
2B	11.7 ± 0.8	1 fixed	495 ± 65	n.a.	n.a.
3A	9.1 ± 0.3	1 fixed	293 ± 65	0.62 ± 0.06	49 ± 40
3B	10.1 ± 0.6	1 fixed	445 ± 78	n.a.	n.a.
4A	10.7 ± 0.9	1 fixed	465 ± 53	0.60 ± 0.07	93 ± 39
4B	11.0 ± 0.5	1 fixed	736 ± 64	n.a.	n.a.

n.a., not applicable.

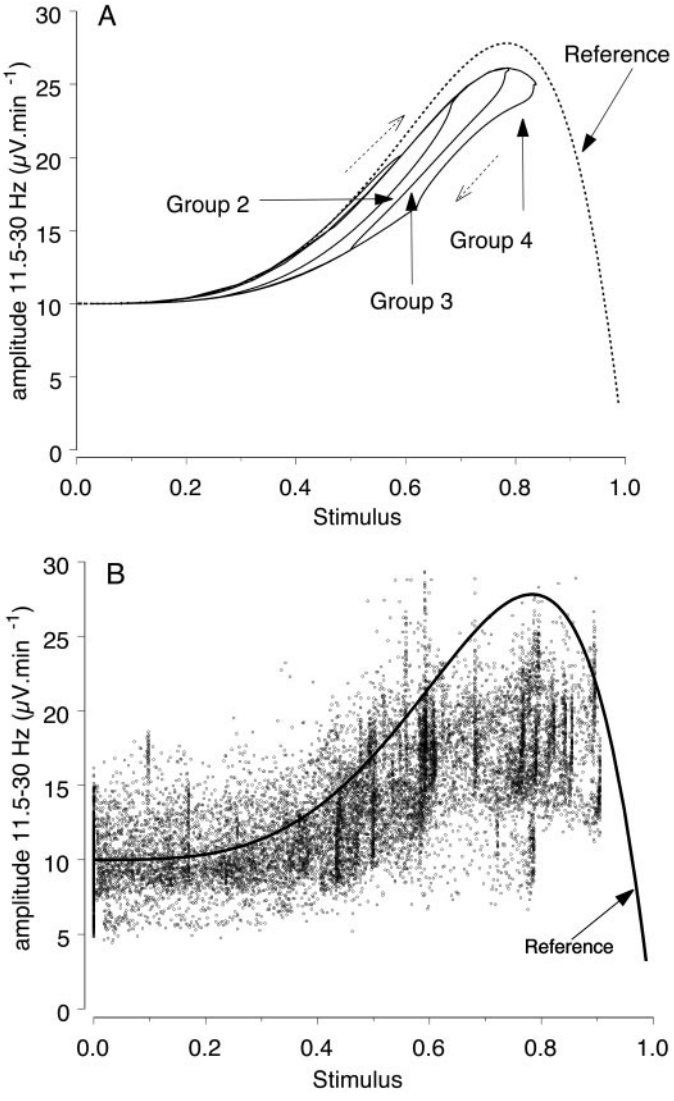

Fig. 8. Simulations (A) and observed (B) stimulus-effect relationship upon combined administration of midazolam and alphaxalone. In A, the proteresis in the stimulus-effect relationship is simulated for all treatment groups A. The reference line represents the stimulus-response relationship for alphaxalone upon short i.v. administration. The dotted arrows indicate the direction of the proteresis. B, dots represent the observed amplitudes of all individuals and the line represents the "reference" stimulus-response relationship for alphaxalone upon short i.v. administration.

effect upon short duration infusion. The duration of the alphaxalone effect was maximal 60 min (data not shown).

Discussion

In the present investigation, the in vivo interaction between the synthetic neuroactive steroid alphaxalone and the benzodiazepine midazolam was characterized and quantified using an integrated mechanism-based PK/PD approach. The pharmacodynamic interaction was best characterized by a model for independent drug-drug interaction. Functional adaptation was observed upon continuous infusion of alphaxalone. By mechanism-based PK/PD analysis, it was shown that the in vivo intrinsic efficacy and affinity at the receptor was not altered to a great extent, but that the transducer function changed substantially over the time course of the experiment. This change was reflected in a change in the height of the stimulus-effect relationship. The reduction in the height of the stimulus-effect relationship did not affect the baseline value at the end of the experiment consistent with the observations.

To characterize the nature of the interaction between two drugs, it is important to characterize the time course of the combined pharmacological effects in combination with the time course of the concentrations to discriminate between pharmacokinetic and pharmacodynamic interactions. In this investigation, no pharmacokinetic interaction was found. The pharmacokinetic parameters of alphaxalone were in agreement with previous investigations (Visser et al., 2002a). The obtained steady-state levels of alphaxalone were close to the predicted levels based on the pharmacokinetic information obtained from short intravenous alphaxalone infusions (Visser et al., 2002a). In agreement with previous observations, it was found that body weight was an important covariate for the pharmacokinetics of alphaxalone. In the present study, little information on the elimination phase was obtained due to the low plasma concentrations and the rapid decrease below the detection limit of the assay. Therefore, the exponent on body weight was fixed at the previously reported value (Visser et al., 2002a). The volume of distribution was slightly higher than for short infusion, presumably due to some accumulation in the fatty tissue in steady-state conditions (Pastorino et al., 1979). The midazolam pharmacokinetic profiles were also in agreement with previous re-

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ports (Mandema et al., 1991; Cleton et al., 1999; Tuk et al., 1999; Visser et al., 2003a). The pharmacokinetic profiles of midazolam were not altered in the presence of alphaxalone and vice versa, indicating the absence of pharmacokinetic interaction.

Pharmacodynamic interactions can occur at the level of drug-receptor interaction but also somewhere in the cascade leading to the pharmacological effect. The interaction between benzodiazepines and neuroactive steroids is of a complex nature. Neuroactive steroids bind to a site that is distinct from the benzodiazepine site (Lambert et al., 1995), although electrophysiological studies indicate that they are functionally coupled (Hawkinson et al., 1994). Furthermore, neuroactive steroids and benzodiazepines can allosterically modulate the $GABA_A$ receptor by increasing binding affinity for GABA and each other, which may account for observed synergistic interaction in vitro (Hawkinson et al., 1994). In the case of allosteric modulation in vivo, the K_{PDA} and K_{PDM} were expected to decrease in the presence of each other. However, no evidence was found in the present investigation that significant allosteric modulation between alphaxalone and midazolam occurred in vivo.

For the interaction between alphaxalone and midazolam, an independent interaction model was chosen, which was first proposed by Bliss (1939) and further described by Ariëns and Simonis (1964). An important characteristic is that a stimulus cannot exceed the maximal stimulus that is achievable in the system. As noted above, the compounds do not possess a similar binding site but produce the same pharmacological effect and thus converge after the receptor activation into the stimulus.

In the present investigation, the effects of both alphaxalone and midazolam were lower than expected due to the development of functional adaptation. Simulations suggested that the functional adaptation was due to an altered stimulus-response relationship. This was supported by the pattern observed in group 4A, where E_{top} seems to be around 20 μV in contrast to 30 to 35 μ in short intravenous administration (Visser et al., 2002a). Only with a reduced height of the parabola could the characteristic biphasic patterns that were observed during the highest dose be described.

The question remains what process caused the reduced stimulus-response relationship. Adaptation can result from a decrease in the number of receptors, sequestration of the receptor away from the outer surface of the membrane, uncoupling of the receptor and its effector unit, or through an adaptation of the second messenger system. In in vitro investigations, it has been reported that GABA enhancement by diazepam binding was decreased due to uncoupling of the binding sites from GABA, by change in phosphorylation of the receptor or other conformational state (Holt et al., 1999). Furthermore, upon chronic treatment of neurosteroids a reduced maximal response was reported for the GABA induced influx by benzodiazepines, pentobarbital, and neurosteroids (Yu and Ticku, 1995; Yu et al., 1996). In addition, chronic exposure to neuroactive steroids resulted in uncoupling of benzodiazepines, neurosteroids, and GABA sites in neuronal cultures (Friedman et al., 1993). However, the results in this investigation suggest that the functional adaptation is not a result of receptor down-regulation but rather an altered stimulus-response relationship. However, this remains to be confirmed in further investigations. To our knowledge, the present study is the first one to report acute pharmacodynamic tolerance development for alphaxalone, although for chronic treatment (more than 1-day administration), tolerance has been reported for minaxolone (Marshall et al., 1997) and allopregnanolone (Czlonkowska et al., 2001).

It would be of interest to investigate the interaction in a reversed design to further characterize the shape of the total stimulus relationship during interaction between midazolam and alphaxalone and to confirm the results obtained in this investigation. Figure 9 shows simulations of a design with steady state concentrations of 0, 15, 30, and 50 ng $\cdot$ ml^{-1} midazolam and a 5-min infusion of 10 mg $\cdot$ kg^{-1} alphaxalone. It is predicted that alphaxalone will exert full effects on each steady-state level with increasing duration, but that the E_{top} will be reduced in case of functional adaptation. Although not investigated, pharmacodynamic adaptation might also occur

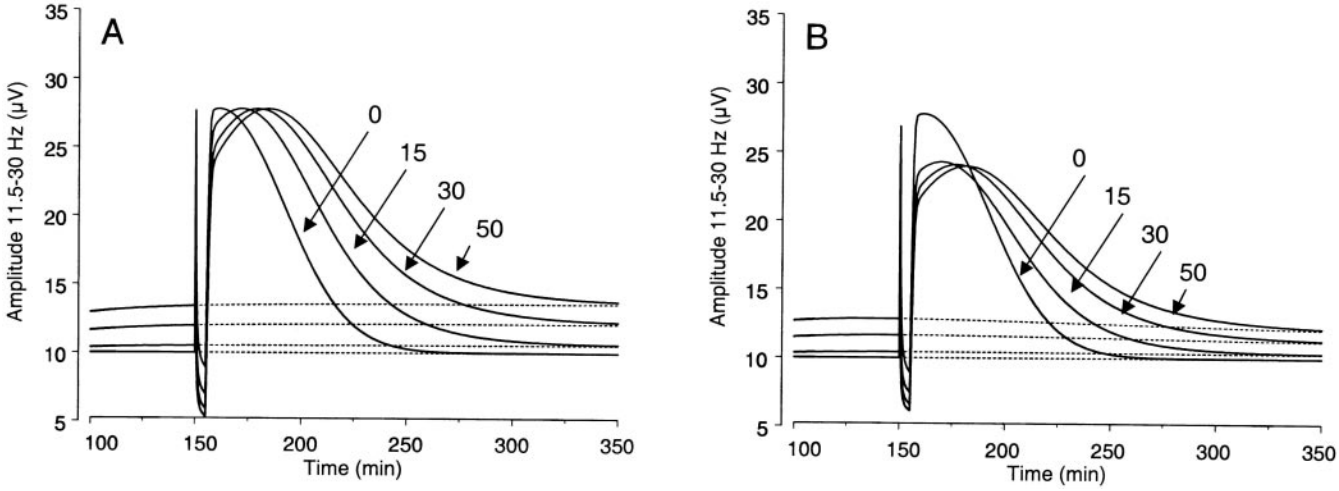

Fig. 9. Simulations of predicted effect profiles of the inverse design of the experiment. For simulations, steady-state concentration levels of midazolam were assumed at 0, 15, 30, and 50 ng $\cdot$ ml^{-1}. Upon steady state, a 5-min infusion of 10 mg $\cdot$ kg^{-1} alphaxalone was simulated. In A, the effects are shown, when midazolam does not induce functional adaptation, whereas in B, it is simulated that midazolam can induce similar functional adaptation as found in the present experiment. It is predicted that increasing concentrations of midazolam will increase the duration of the alphaxalone effects. When functional adaptation might occur, this will be observed in a reduced E_{top} of the alphaxalone effect compared with vehicle treatment. However, it is predicted that alphaxalone will still induce a full biphasic effect profile.

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due to prolonged midazolam administration. In a design with steady-state levels of midazolam and a bolus allopregnanolone, the concentration-EEG effect relationship of allopregnanolone was decreased in height compared with control treatment (Bart Laurijssens, personal communication), although no functional adaptation was reported for EEG effects upon chronic administration of midazolam (Laurijssens and Greenblatt, 2002).

In conclusion, using an integrated mechanism-based PK/PD modeling approach, the independent interaction between midazolam and alphaxalone was quantified. No evidence was found for the presence of allosteric modulation. The interaction resulted in an elevation of the midazolam effect upon alphaxalone treatment, which was much less than expected due to the development of functional adaptation. Although either adaptation in the stimulus-response relationship or desensitization in drug-receptor interaction could describe the effects of alphaxalone upon continuous administration, only an altered transducer function was able to account for the observed patterns for the combined administration of midazolam and alphaxalone.

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