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Characterization of the Pharmacokinetic and Pharmacodynamic Interaction between Gamma-Hydroxybutyrate and Ethanol in the Rat

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It has been reported that ethanol enhances the hypnotic effect of gamma-hydroxybutyrate (GHB). In order to clarify the nature of this interaction we studied the pharmacokinetics and pharmacodynamics of combinations of GHB and ethanol in rats. Intraperitoneal injections of the GHB precursor gamma-butyrolactone (300 mg/kg) together with ethanol (3000 mg/kg) ($n = 4$) resulted in a longer "sleeping time" than the sum of the individual times ($n = 8$). Pharmacokinetic analysis of GHB concentrations with a two-compartment model with Michaelis-Menten (M-M) elimination in rats receiving a bolus of GHB (400 mg/kg, i.v.) in addition to steady-state ethanol concentrations (300–3000 μg/ml) ($n = 12$) or saline ($n = 15$) showed no marked differences in the area under the curve. The nature of the pharmacodynamic interaction was studied using isobolographs and an interaction model for the loss of the startle and righting reflex and a reaction to a painful tail clamp in rats receiving combinations of steady state concentrations of ethanol (1000–3000 μg/ml) and GHB (200–1400 μg/ml). For the righting reflex, synergy was observed at high ethanol concentrations (>2000 μg/ml) and additivity at lower concentrations. For the startle reflex, it was antagonistic at ethanol concentrations below 1000 μg/ml, and additivity was seen at higher concentrations. For the tail clamp reaction, a slight but significant antagonism was found at all combined concentrations. It is concluded that ethanol prolongs the sleeping time induced by GHB in the rat, which may not be due to a pharmacokinetic interaction. Pharmacodynamic interactions between GHB and ethanol in the rat occur, and the nature varies with the reflex studied and the concentration of ethanol used.

Key Words: **gamma-hydroxybutyrate; GHB; ethanol; pharmacokinetics; pharmacodynamics; interaction; additivity; synergy; illicit drugs; recreational drugs.**

Gamma-hydroxybutyrate (GHB) is a naturally occurring substance with neuromodulating properties (Maitre, 1997). After peripheral administration it crosses the blood-brain barrier and, in high doses, induces behavioral responses including sedation and anesthesia (Cash, 1994). Although GHB is nowadays only sporadically used in anesthesia (Kleinschmidt *et al*., 1998), it recently has gained attention as an investigational sedative in intensive care patients (Kleinschmidt *et al*., 1999; Soltész *et al*., 2001) and has been investigated in the treatment of alcohol dependence (Gallimberti *et al*., 2000) and narcolepsy (Scrima *et al*., 1990). Furthermore, GHB is increasingly misused as a recreational drug, with high doses leading to deep coma and even death (Chin *et al*., 1998; Zvosec *et al*., 2001). Recent case series have shown that a large number of the patients presenting in coma after a GHB intoxication have coingested other drugs such as amphetamines, cocaine, and ketamine, and frequently also ethanol (Mason and Kerns, 2002; Miro *et al*., 2002; Sporer *et al*., 2002; Van Sassenbroeck *et al*., 2002b). It has been suggested that ethanol enhances the sedative effect of GHB in humans (Couper and Marinetti, 2002; Mattila *et al*., 1978; Nicholson and Balster, 2001), and animal research has shown that coadministration of GHB and ethanol induces a longer sleeping time than the sum of the sleeping times induced by the individual substances (McCabe *et al*., 1971). This potentiation can theoretically be explained by a pharmacokinetic interaction resulting in an increased effect site concentration of one or both of the drugs or by a pharmacodynamic interaction at the effect sites. A pharmacokinetic interaction due to a decrease in the metabolism of GHB by ethanol has been suggested (Couper and Marinetti, 2002), based on measurements of the concentration of endogenous GHB after peripheral ethanol administration (Poldrugo and Snead, 1984, 1985; Roth, 1970). For exogenously administered GHB, however, it is unclear whether coadministration with ethanol results in increased GHB or ethanol plasma concentrations (Nicholson and Balster, 2001), and we therefore decided to study the pharmacokinetics. In the present experiments, we also studied a possible pharmacodynamic interaction between ethanol and GHB. The depth of sedation or hypnosis induced by both substances was determined by the recording of physiological responses to different stimuli (Prys-Roberts, 1987). These stimulus-response measures were related to the plasma concentrations of GHB and ethanol (Bol *et al*., 1999).

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MATERIALS AND METHODS

Animals and Animal Instrumentation

The study protocol was approved by the ethical committee for animal research of the Faculty of Medicine of the University of Ghent, Belgium. Male Wistar rats (374 ± 5 g), 10–12 weeks old were purchased from Iffa Credo (L'Arbresle Cedex, France), kept at 21 ± 1°C with an air humidity of 55 ± 3%, and fed a standard laboratory diet (Pavan, Oud-Turnhout, Belgium) in a 12 h : 12 h light-dark cycle. Three rats were housed per cage (60 × 40 × 20 cm) on wooden chips. The day before the experiment, the animals were housed individually in cages (40 × 25 × 20 cm) to avoid damage to the catheters exteriorized at the nape of the neck. The rats were deprived of food 12 h before the start of the experiments. In each experiment, the observer was blinded to the treatment and all experiments started between 8 and 9 A.M.

One day before the experiment, polyethylene catheters (PE 10) filled with heparine solution (100 IU/ml) were inserted into the femoral artery and vein and the jugular vein and exteriorized at the nape of the neck. All surgery was carried out under pentobarbital anesthesia (60 mg/kg intraperitoneally). The arterial line was used for blood sampling, the jugular vein for the infusion of ethanol, and the femoral vein for the infusion of GHB. In order to minimize restraining stress during the experiment, the animals were put in the restraining cage on several occasions before the actual experiment. It should be remarked that during the experiment each animal was kept as shortly as possible in this restraining cage. Once the animal was enough sedated to avoid damaging of the intravascular lines, it was taken out of the restraining cage and placed in its individual cage on wooden chips.

During the experiment, the core temperature was measured every hour with a flexible thermistor probe inserted rectally to a depth of 5 cm, and a heating lamp externally warmed the animal when the temperature fell below 37°C.

Experimental Protocol

Three series of experiments were conducted. In the first series, the sleeping times induced by one dose of the GHB precursor gamma-butyrolactone (GBL), ethanol, and their combination were measured. In the second series, the pharmacokinetic parameters of a bolus of GHB were studied in four different groups under steady state infusions of different concentrations of ethanol and compared with a steady state infusion of saline. In a third series, the pharmacodynamics of GHB were determined in three groups in the presence of different steady state infusions of ethanol and compared with a steady-state infusion of saline.

First Series of Experiments

Three groups ($n = 4$) of rats were treated intraperitoneally respectively with ethanol (Merck Eurolab, Leuven, Belgium, 3000 mg/kg, 250 mg/ml saline) and saline (group 1); the GHB precursor GBL (Sigma, St. Louis, MO, 300 mg/kg, 150 mg/ml saline) and saline (group 2); or a combination of the same doses of GBL and ethanol (group 3). The GHB precursor GBL was used for intraperitoneal injection instead of GHB due to its more rapid onset of action and more predictable dose response (Snead, 1991). GBL is rapidly and completely hydrolyzed in the blood to GHB by a lactonase (Roth and Giarman, 1966) and has no activity in the brain (Snead, 1991).

The time between loss and return of the righting reflex was measured in all rats. At return of the righting reflex, a blood sample (100 μl) was taken from the tail to measure the plasma concentrations of the two drugs.

Second Series of Experiments: Pharmacokinetic Study

In this series the influence of different steady-state plasma concentrations of ethanol on the pharmacokinetics of GHB was studied in four groups of rats. The various steady-state ethanol concentrations targeted were 300 μg/ml (group 1, $n = 4$), 1000 μg/ml (group 2, $n = 4$), 2000 μg/ml (group 3, $n = 4$), and 3000 μg/ml (group 4, $n = 4$). A control group received a continuous infusion of saline instead of ethanol (group 5, $n = 15$).

Loading doses and infusion rates of ethanol were calculated using equations from literature data (Gibaldi and Perrier, 1982). The values for the pharmacokinetic parameters used to calculate the loading dose and the infusion rate were based on results of a pharmacokinetic analysis using ethanol bolus doses in preliminary experiments.

Forty-five min after the start of the steady-state infusion of ethanol, a single bolus of GHB (Sigma, St. Louis, MO, 133 mg/ml water) was given at a rate of 400 mg/kg over 5 min. Arterial blood samples were taken as a function of time for determination of GHB (100 μl) and ethanol plasma concentrations (50 μl). Sampled blood was replaced with the same amount of isotonic saline solution. To rule out the possibility that the potentiation of the hypnotic effect could be explained by increases in the ethanol concentration due to an inhibition of its metabolism, we also administered a bolus of ethanol (1000 mg/kg over 5 min) during a continuous infusion of GHB ($n = 4$), targeting 300 μg/ml, or saline ($n = 4$). No differences in the pharmacokinetic parameters of ethanol were observed (data not shown).

Third Series of Experiments: Pharmacodynamic Study

In this series the relationship between various stimulus-response measures (Gustafsson *et al*., 1996) and different steady-state concentrations of ethanol and GHB was studied by pharmacokinetic-pharmacodynamic modeling in five different groups of rats. The pharmacokinetics and pharmacodynamics of GHB and/or ethanol were investigated simultaneously in the same experiment for each animal.

Pharmacokinetics. Three groups of rats ($n = 4$) received an infusion of ethanol targeting steady state concentrations of 1000 μg/ml (group 1), 2000 μg/ml (group 2), and 3000 μg/ml (group 3). At six different time points during the experiment (20, 30, 35, 90, 190, and 290 min after the start of the ethanol infusion), a blood sample was taken for measurement of the actual ethanol concentrations.

Forty-five min after the start of the ethanol infusion, a GHB infusion was given, targeting in a sequential way the following steady-state GHB concentrations: 200, 400, 600, 800, 1000, 1200, and 1400 μg/ml. In order to assess the effects of GHB or ethanol alone, two control groups were randomly included. In the first control group (group 4, $n = 6$), a continuous infusion of saline was administered, and the same sequential GHB concentrations were targeted as for groups 1, 2, and 3. In the second control group (group 5, $n = 6$), a continuous infusion of saline was administered, and the following ethanol concentrations were subsequently targeted: 500, 1000, 2000, 3000, 4000, and 5000 μg/ml. Each target concentration was maintained for 50 min before targeting a subsequent concentration. At each concentration level, three arterial blood samples were taken at 20, 30, and 45 min after this new concentration level was targeted, to determine the actually achieved plasma concentrations of GHB or ethanol.

Loading doses and infusion rates of ethanol and GHB were calculated using previously published equations (Gibaldi and Perrier, 1982), and the pharmacokinetic parameters from the second series of experiments were used to calculate the loading dose and the infusion rates.

Pharmacodynamics. Our previous research of the hypnotic effect induced by GHB showed that there is a distribution-delay (hysteresis) between the plasma and the effect-site concentration in the brain with a half-life of about 5 min (Van Sassenbroeck *et al*., 2001, 2002a). To avoid this hysteresis, we allowed the effect site concentrations to equilibrate with the steady-state plasma concentrations of GHB by leaving a 20-min period (four times the distribution half-life) before measuring the responses to four stimulus-response measures. These stimulus-response measures were determined according to a protocol published by Bol *et al*. (1999, 2000) and Gustafsson *et al*. (1996). In brief, 20 min after targeting each new concentration the following stimulus-response measures were tested: the whisker reflex (WR), the startle reflex to a hand clap (SR), and the righting reflex (RR) as measures of the hypnotic effect of GHB or ethanol. We also measured the tail clamp reaction (TC) as a measure of their anaesthetic action. A positive whisker reflex was defined as a purposeful movement of the head towards the side where the whiskers were stroked with a fine needle. A positive righting reflex was defined as spontaneous return of the rat on its four paws within 15 seconds when placed on its

back. A hand clap, out of sight of the rat, was used to assess the presence of the startle reflex, which was defined as any movement of the body upon the sudden noise. For the tail clamp, a clamp was placed on the tail for a maximal time of 15 seconds. Any movement of the body was defined as a positive reaction. The location of the stimulus was marked to avoid the use of previously used portions of the tail. To avoid excessive stimulation, only some of these reflexes were tested at each target concentration. At low concentrations, only the whisker, startle, and righting reflexes were tested, and the tail clamp reaction was only tested after two of the other reflexes had disappeared. This means that a window of three (or less) of the stimulus-response measures moved along the concentration curve. Each stimulus-response measure was measured four times per concentration level. Once a response was lost, it was reexamined at the subsequent higher concentration level, and if it remained negative, it was assumed to be negative at all subsequent concentrations. The stepwise increase in GHB or ethanol concentrations was stopped before the maximal target concentration was reached when all stimulus-response measures had disappeared. The average of the plasma concentrations of the three blood samples taken at each targeted concentration level was used for the correlation with the pharmacodynamic measures. This procedure led to a total of between 32 and 52 concentration-response pairs for each rat. A positive response to a stimulus was assigned a value of one (Y = 1), and a negative response to a stimulus was assigned a value of zero (Y = 0).

Data Analysis

The pharmacokinetics of GHB in the second series of experiments was quantified as described earlier (Van Sassenbroeck *et al*., 2001). In brief, a two-compartmental model with Michaelis-Menten (M-M) elimination kinetics was fitted to the plasma concentration-time profiles of each individual rat using Winnonlin version 1.5 (Scientific Consulting, Inc):

$$\frac{dC_1}{dt} = \frac{R}{V_C} - \frac{Cl_d \cdot C_1}{V_C} + \frac{Cl_d \cdot C_2}{V_T} - \frac{V_{\max} \cdot C_1}{(K_m + C_1) \cdot V_C} \quad (1)$$

$$\frac{dC_2}{dt} = \frac{Cl_d \cdot C_1}{V_C} - \frac{Cl_d \cdot C_2}{V_T} \quad (2)$$

where dC_1/dt is the rate of decline of drug concentration at time t, V_C the distribution volume of the central compartment, V_T the distribution volume of the peripheral compartment, R the infusion rate, Cl_d the intercompartmental clearance, C_1 the concentration in the central compartment, C_2 the concentration in the peripheral compartment, V_{max} the theoretical maximum rate of the elimination, and K_m the M-M constant. The steady-state volume of distribution (V_{dSS}) was defined as the sum of the V_C and the V_T.

A two-compartment model proved to be the best fitting model based on the Akaike Information Criterion (AIC) (Akaike, 1974), the visual inspection of the curve, and the residual plots (Gabrielsson and Weiner, 1997). Using the estimated pharmacokinetic parameters, plasma concentration-time curves were constructed from time zero to a common final time point of 300 min because the time of the last measurable sampling point varied. The area under the curve (AUC) from time 0 to 300 min was then calculated using the trapezoidal rule (Kinetica 2000, Innaphase Co., Philadelphia, USA).

In the second and third series of experiments, the prediction error (PE) for each pair of predicted and measured plasma concentrations was calculated as (Kazama *et al*., 1998):

$$PE = \frac{C_p(measured) - C_p(predicted)}{C_p(predicted)} * 100 \quad (3)$$

with C_p the plasma concentration. The median prediction error was calculated.

The stimulus-response results of the individual rats (third series) were pooled for each treatment group. The pooled "response" (Y = 1) and "no response" (Y = 0) data for each stimulus-response measure were converted into a continuous probability versus drug concentration relationship via logistic regression (Bol *et al*., 1999). The concentration level where both positive and negative answers to a certain stimulus were measured was incorporated in the analysis. The probability to measure no response to a certain stimulus was determined using the following equation (Bol *et al*., 2000; Greco *et al*., 1995; Mandema *et al*., 1992):

$$P(Y = 0/C_e, C_g) = \frac{\left(\frac{C_g}{EC_{50G}} + \frac{C_e}{EC_{50E}} + S * \frac{C_g}{EC_{50G}} * \frac{C_e}{EC_{50E}}\right)^N}{\left(\frac{C_g}{EC_{50G}} + \frac{C_e}{EC_{50E}} + S * \frac{C_g}{EC_{50G}} * \frac{C_e}{EC_{50E}}\right)^N + 1} \quad (4)$$

This is a general interaction model for two drugs, which induce the same pharmacological effect via different receptor sites, without specific knowledge of the mechanism of action of the drugs. P is the probability of no response to the stimulus (Y = 0) at the steady-state GHB concentration C_g and a certain ethanol concentration C_e. EC_{50G} and EC_{50E} are, respectively, the GHB and ethanol concentrations where 50% of the stimulus-response answers were negative when either GHB (group 4) or ethanol (group 5) was given alone. N is a measure of curve steepness and S is the interaction factor that expresses the nature and extent of the drug interaction. The interaction between GHB and ethanol is synergistic when $S > 0$, additive when $S = 0$, and antagonistic when $S < 0$. For both a synergic or antagonistic interaction it is held that the larger S differs from zero, the larger the extent of the interaction will be.

When the concentration of one of both drugs is constant, Equation 4 can be simplified into Equation 5 (Bol *et al*., 2000; Mandema *et al*., 1992):

$$P(Y = 0/C_e, C_g) = \frac{C_p^N}{C_p^N + EC_{50p,APP}^N} \quad (5)$$

where C_p is the plasma concentration of the drug of which the concentration changes over time and $EC_{50p,\ APP}$ is the apparent EC_{50} of that drug. This $EC_{50p,\ APP}$ will be equal to the EC_{50} of the drug when the concentration of the other drug is zero. The value of the $EC_{50,\ APP}$ alone, however, does not discriminate between synergistic, additive, or even antagonistic interactions when this $EC_{50,\ APP}$ is smaller than the EC_{50}. The nature of the interaction can only be determined by means of Equation 4 and the estimated S value, or by constructing 50% isoboles with (on the Y-axis) the GHB concentration needed to abolish 50% of the positive answers to a stimulus (EC_{50G}) when GHB is administered alone (group 4), and (on the X-axis) the ethanol concentration needed to abolish 50% of the answers (EC_{50E}) when ethanol is administered alone (group 5). For the three steady state ethanol concentrations targeted (group 1, 2 and 3), the $EC_{50,\ APP}$ of GHB needed to abolish 50% of the positive answers to the stimuli were placed on the GHB-ethanol iso-effect field. A 50% isobole was fitted to these five data points with the following equation (Berenbaum, 1989; Kazama *et al*., 1998):

$$\frac{C_g}{EC_{50G}} + \frac{C_e}{EC_{50E}} + \varepsilon * \frac{C_g}{EC_{50G}} * \frac{C_e}{EC_{50E}} = 1 \quad (6)$$

where ε corresponds to the S parameter in Equation 4. The fit of this 50% isobole was judged based on the AIC (Akaike, 1974), the visual inspection of the curve and the residual plots (Gabrielsson and Weiner, 1997). It proved satisfactory only for the tail clamp reflex, as the iso-effective line of the startle and the righting reflex crossed the line of additivity. For these two reflexes, the nature and extent of the interaction was interpreted by calculating the S value of each individual $EC_{50,\ APP}$ data point by means of Equation 4.

Drug Assay

GHB was determined in rat plasma (60 μl) by a validated high-pressure liquid chromatography method as described previously (De Vriendt *et al*., 2001). The calibration curve ranged from 10 to 750 μg/ml GHB. Quality control samples at low (20 μg/ml), medium (300 μg/ml), and high (700 μg/ml) concentrations were analyzed in duplicate together with the samples. For each quality control sample, the coefficient of variation was <13% (n = 12), and

the accuracy was between 96% and 99% ($n = 12$). The lower limit of quantitation was 10 μg/ml.

Ethanol was determined in rat plasma (20 μl) by an Alcohol Determination kit 332-B (Sigma Diagnostics, St. Louis, MO). The calibration curve ranged from 50 to 3000 μg/ml. A quality control sample provided by the determination kit at 800 μg/ml was analyzed in duplicate together with the samples. For the quality control sample, the coefficient of variation was $< 5\%$ ($n = 15$), and the accuracy was between 96 and 113% ($n = 15$). The lower limit of quantitation was 50 μg/ml.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). The sleeping time of the rats receiving both GHB and ethanol was compared to the sum of the individual sleeping times with a Student *t*-test. The pharmacokinetic parameters of the different treatment groups were compared with a one-way ANOVA, followed by a Newman-Keuls test if necessary (Statistica version '99, Statsoft Inc., Tulsa, OK). Fifty percent isoboles were constructed for the stimulus-response measures. The possibilities of either an additive or a non-additive interaction were examined by unweighted least squares nonlinear regression analysis with the value of ε estimated equal to or different from 0 (Equation 6). The residual sum of squares (RSS) of both fitted curves were compared with an *F*-test to determine which fitted line correlated best with the original data (Vuyk *et al*., 1995). For the startle reflex and the righting reflex, *S* values for the individual EC_{50} data points were calculated using Equation 4. These *S* values were considered different from zero when their 95% confidence interval did not encompass zero (Greco *et al*., 1995; Salonen *et al*., 1992); $p < 0.05$ was considered as statistically significant.

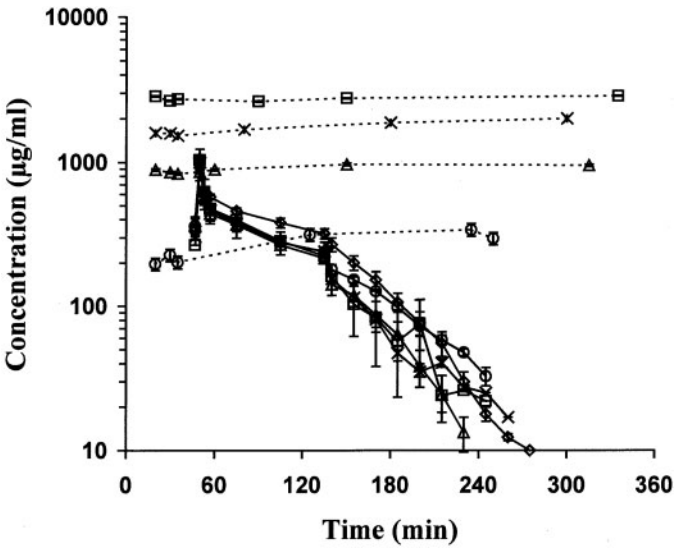

FIG. 1. Mean (± SEM) GHB (—) and ethanol (- - - -) plasma concentration time curves after intravenous infusion of GHB (400 mg/kg during 5 min) in GHB-alone controls ($n = 15$, ◇), and rats receiving a continuous infusion of ethanol targeting 300 μg/ml ($n = 4$, ○), 1000 μg/ml ($n = 4$, △), 2000 μg/ml ($n = 4$, X) and 3000 μg/ml ($n = 4$, □). The GHB infusion (400 mg/kg over 5 min) started at time 45 min.

RESULTS

First Series of Experiments

The sleeping time of rats receiving both GBL and ethanol (389 ± 6 min) was significantly longer than the sum of the sleeping times in the rats receiving the same doses of only ethanol (231 ± 9 min) or GBL (66 ± 4 min) ($p < 0.0001$). The ethanol and GHB concentration at return of the righting reflex of the rats receiving both drugs were 1906 ± 144 μg/ml and 196 ± 37 μg/ml respectively. The ethanol and GHB concentration at return of the righting reflex were 2732 ± 138 μg/ml and 544 ± 21 μg/ml respectively when both substances were given separately.

Second Series of Experiments: Pharmacokinetic Study

Figure 1 shows the mean GHB and ethanol versus time curves in rats receiving a steady state infusion of ethanol (groups 1, 2, 3, and 4) and in controls receiving a steady-state infusion of saline (group 5). The ethanol concentrations reached a steady state with a slight but nonsignificant increase over time. This small increase over time of all steady-state ethanol concentrations was also observed when a bolus of saline was given instead of GHB ($n = 4$, data not shown). The measured mean ethanol concentrations were slightly lower than targeted (263 ± 28 μg/ml instead of 300 μg/ml, 889 ± 14 μg/ml instead of 1000 μg/ml, 1659 ± 64 μg/ml instead of 2000 μg/ml, and 2745 ± 42 μg/ml instead of 3000 μg/ml), resulting in median prediction errors of –13%, –11%, –13%, and –9% for the target ethanol concentrations of 300, 1000, 2000, and 3000 μg/ml, respectively. No major differences in the overall course of the different GHB concentration versus time curves were observed. The calculated pharmacokinetic parameters for a two-compartment model with M-M elimination kinetics are given in Table 1. There were no significant differences for the AUC between the different treatment groups. The maximal metabolic rate (V_{max}) was significantly lower in the ethanol-treated groups ($p = 0.03$, ANOVA), but no between-groups differences could be detected (Newman-Keuls post-hoc test). In rats receiving a continuous infusion of ethanol, an increase in peripheral volume of distribution (V_T) ($p = 0.03$, ANOVA) was observed, resulting in a statistically significant increase in steady-state volume of distribution (V_{dSS}) ($p = 0.006$). Post-hoc comparisons for between-group differences only revealed a significant difference between the ethanol 300 μg/ml-group and the control group for the V_{dSS} ($p = 0.015$, Newman-Keuls).

Third Series of Experiments: Pharmacodynamic Study

The measured mean ethanol concentrations were slightly lower than targeted (944 ± 45 μg/ml instead of 1000 μg/ml, 1749 ± 40 μg/ml instead of 2000 μg/ml, and 2464 ± 47 μg/ml instead of 3000 μg/ml were measured), which was reflected in median prediction errors of –6%, –13%, and –18% for the target ethanol concentrations of 1000, 2000, and 3000 μg/ml, respectively. Analysis of variance showed no significant changes in the measured ethanol concentrations during the experiment.

The measured GHB concentrations of 1000 μg/ml and higher were comparable with their corresponding targeted concentrations with a median prediction error of –2%. For the

TABLE 1
Pharmacokinetic Parameters of GHB after an Intravenous Infusion of 400 mg/kg over 5 Min in Controls Receiving Saline and Rats Receiving a Continuous Infusion of Ethanol

	Control (n = 15)	Ethanol 300 (n = 4)	Ethanol 1000 (n = 4)	Ethanol 2000 (n = 4)	Ethanol 3000 (n =4)
V_{max} (μg/kg/min)[a]	3421 ± 208	2539 ± 421	2303 ± 115	2889 ± 227	2590 ± 150
K_M (μg/ml)	75 ± 12	74 ± 37	39 ± 12	66 ± 14	40 ± 17
V_C (ml/kg)	263 ± 23	273 ± 29	279 ± 17	329 ± 44	244 ± 28
V_T (ml/kg)[a]	427 ± 37	668 ± 65	540 ± 16	479 ± 63	514 ± 53
$V_{d\ SS}$ (ml/kg)[a]	690 ± 33[b]	940 ± 66[b]	819 ± 27	807 ± 19	758 ± 43
Cl_d (ml/min/kg)	63 ± 7	53 ± 2	47 ± 6	46 ± 1	47 ± 12
$AUC_{0\text{-}300}$ (mg.min/ml)	44 ± 2	46 ± 2	50 ± 2	43 ± 4	47 ± 4

Note. Rat ethanol infusions targeted 300, 1000, 2000, and 3000 μg/ml. V_{max} is the theoretical maximum rate of the elimination, K_m the M-M constant, V_C the distribution volume of the central compartment, V_T the distribution volume of the peripheral compartment, V_{dSS} the steady-state volume of distribution defined as $V_C + V_T$, Cl_d the intercompartmental clearance and AUC the area under the curve extrapolated from time 0 to 300 min. Results are expressed as mean ± SEM.

[a] $p < 0.05$, one-way ANOVA.

[b] $p < 0.05$ post-hoc comparison with Newman-Keuls test.

lower target GHB concentrations, the measured concentration was larger than expected, with a large median prediction error of 45% for a target GHB concentration of 200 μg/ml. This median prediction error gradually decreased to 19% for the target concentration of 800 μg/ml. No significant differences were found, however, between the four drug regimens at each target concentration.

The administration of GHB (group 4) and ethanol (group 5) alone produced a concentration-dependent disappearance of the whisker, startle, and righting reflexes and the tail clamp reaction (Fig. 2, Table 2). The sequence of disappearance is different, as with GHB (group 4) the startle reflex disappeared before the righting reflex, and for ethanol (group 5) the rats lost the righting reflex before the startle reflex. The whisker reflex disappeared already in 70% of the rats receiving the continuous infusion of ethanol targeting 1000 μg/ml (group 1), which precluded further analysis of the interaction between GHB and ethanol.

Figure 2 shows the probability of loss of the startle reflex, the righting reflex, and the tail clamp reaction, respectively, as a function of the GHB plasma concentration in the presence of ethanol targeting 1000 μg/ml (group 1), 2000 μg/ml (group 2), or 3000 μg/ml (group 3) and in comparison with the control groups only receiving GHB (group 4) and ethanol (group 5). For the ethanol concentration of 1000 μg/ml the probability curves of the righting reflex and the tail clamp reaction show a slight shift to the left as compared to the control group only receiving GHB. For the startle reflex, however, a clear shift of the probability curve to the right can be observed. This is also reflected in the $EC_{50,\ APP}$ values (Table 2), which show for the $EC_{50,\ APP}$, a small nonsignificant decrease for the righting reflex, a significant decrease for the tail clamp reaction ($p < 0.05$), and a significant increase in the $EC_{50,\ APP}$ for the startle reflex ($p = 0.001$, Student t-test) when targeting 1000 μg/ml ethanol.

The ethanol concentrations of 2000 and 3000 μg/ml induced a shift to the left of the probability curves of all stimulus-response measures (Fig. 2) and a decrease in the GHB concentration needed to induce a 50% loss of response (Table 2).

Further characterization of the nature and extent of the GHB-ethanol interaction for the different stimulus-response measures was done at the EC_{50} level by constructing isobolograms. These isobolograms display the concentrations of both substances needed to induce a 50% probability of loss of the stimulus-response measure when the drugs are combined (group 1, 2, 3) or given alone (group 4 and 5) (Fig. 3). The isobologram of the tail clamp reaction was fitted with Equation 6 and the estimated value for ε was −0.32 ± 0.02. An F-test revealed a difference between the fitted curve and the line of additivity (RSS 35328 versus RSS 1162, $F = 118$, $p < 0.05$), i.e., a slight antagonism between ethanol and GHB. The course of the isobolograms of the startle and the righting reflex could not be fitted properly with Equation 6 (judged by the Akaike information criterion and the residual plots) and therefore, the extent of interaction at each EC_{50} point was separately calculated with Equation 4. At the ethanol level of 944 ± 45, 1749 ± 40, and 2464 ± 47 μg/ml, S values of –1.49 [–1.67, –1.31], −0.31 [–1.41, 0.79] and 0.61 [–0.02, 1.24] (mean and 95% CI), respectively, were obtained for the startle reflex, suggesting antagonism between GHB and ethanol at the lower concentration of ethanol. The S values for the righting reflex were −0.73 [–1.5, 0.04], 1.07 [–0.15, 2.29] and 10 [0.8, 19.2] respectively, indicating a synergy between both drugs at the higher concentrations of ethanol.

DISCUSSION

GHB is often used together with ethanol at "rave parties" (Louagie *et al*., 1997; Mason and Kerns, 2002; Miro *et al*., 2002; Sporer *et al*., 2002; Van Sassenbroeck *et al*., 2002b), and literature data suggest that high doses of ethanol may potentiate

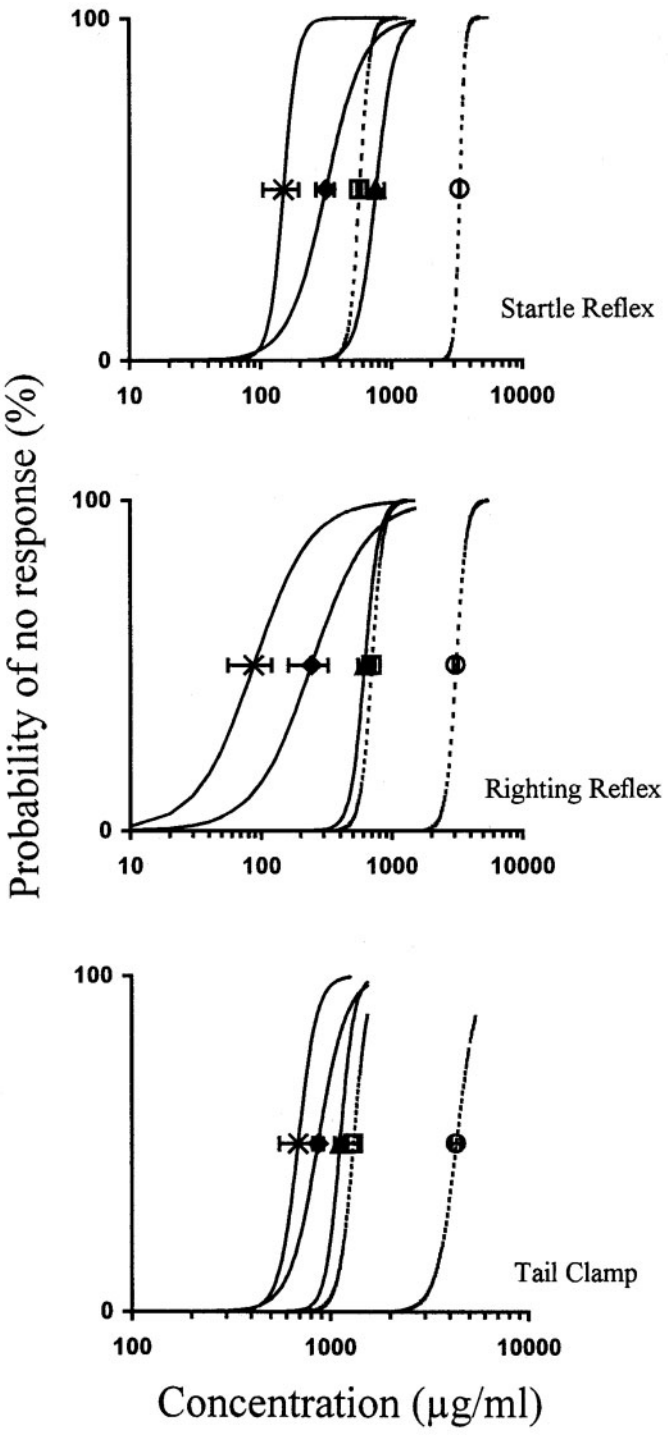

FIG. 2. Continuous relationship between steady state plasma concentrations of GHB and ethanol, respectively, and the probability of no response to three stimulus response measures: startle reflex, righting reflex and tail clamp reaction, for the GHB-alone group (open box, dashed line), the ethanol-alone group (open circle, dashed line), and for increasing concentrations of GHB in the presence of 944 ± 45 μg/ml (solid triangle), 1749 ± 40 μg/ml (solid circle), and 2464 ± 47 μg/ml (x) of ethanol. The symbols represent the $EC_{50,APP}$ value with its 95% confidence interval.

the hypnotic effect of GHB, resulting in an impairment of the coordination and manual proprioception needed as motor driving skills (Mattila *et al*., 1978).

The first aim of the present study was to find out whether the "sleeping time," defined as the time between loss and return of the righting reflex, induced by the combination of GHB and ethanol in the rat was longer than the sum of the sleeping times induced by GHB and ethanol alone, as has been reported by McCabe *et al*. (1971). Our first series of experiments confirms these results for the high doses of both substances used and shows that at the return of the righting reflex the concentrations of both GHB and ethanol in the rats receiving both substances were significantly lower than the concentrations of each substance at return of the righting reflex when administered alone.

These observations, however, cannot prove a synergistic interaction between GHB and ethanol and do not allow a conclusion whether this is explained by a pharmacokinetic and/or a pharmacodynamic interaction. With regard to the pharmacokinetics, previous research in the rat has shown that the administration of ethanol induces an increase in the endogenous GHB concentrations in the liver and/or the brain (Poldrugo and Snead, 1984, 1985; Roth, 1970), due to a competition between ethanol and GHB for the NAD^+-dependent degradation, which may involve alcohol dehydrogenase (Nicholson and Balster, 2001; Poldrugo and Addolorato, 2001). Based on an extrapolation of these results, it has been hypothesized that the potentiation of the hypnotic effect of exogenous GHB or ethanol may be due to an increase in the effect-site concentration of one or both of the drugs (Couper and Marinetti, 2002). We therefore investigated the pharmacokinetics of GHB and ethanol in a second series and the pharmacodynamics in a third series of experiments.

We did not find a consistent influence of a steady-state infusion of ethanol either on the overall course of the GHB plasma concentration versus time curve or on the area under the plasma concentration-time curve. Small increases in V_T and V_{dSS} were observed, which may be due to the fact that ethanol is a peripheral vasodilator that increases V_T, which may lead to an increase in V_{dSS} (Baraona *et al*., 2002; Fazio *et al*., 2001). Ethanol induced a small decrease in the maximal metabolic rate (V_{max}) of GHB, but no between groups differences could be detected.

With regard to the pharmacodynamics, we investigated the influence of different concentrations of both GHB and ethanol on three different stimulus-response measures to characterize the interaction of GHB and ethanol (Prys-Roberts, 1987; Vuyk *et al*., 1995). The control experiments in which GHB and ethanol were administered alone showed a difference in sensitivity for the loss of the stimulus-response measures. Indeed, the molar ethanol concentrations needed to induce 50% loss of the different stimulus-response measures were eight- to tenfold higher than the concentrations needed for GHB. GHB, therefore, is a stronger anaesthetic than ethanol, but it should be noted that the steady-state EC_{50} of GHB is still much larger than the values reported for more common hypnotic drugs like

TABLE 2
Estimated EC_{50} Steady-State Plasma Concentrations for GHB and Ethanol (μg/ml)

	GHB (EC_{50G})	GHB ($EC_{50,\ APP}$)			Ethanol (EC_{50E})
Continuous infusion	Saline ($n = 6$)	Ethanol 1000 μg/ml ($n = 4$)	Ethanol 2000 μg/ml ($n = 4$)	Ethanol 3000 μg/ml ($n = 4$)	Saline ($n = 6$)
Whisker reflex	309 ± 1	—[a]	—[a]	—[a]	855 ± 2
Startle reflex	570 ± 5	762 ± 30[b]	316 ± 13[b]	150 ± 65[b]	3310 ± 5
Righting reflex	698 ± 3	618 ± 6	242 ± 21[b]	29 ± 8[b]	3072 ± 6
Tail clamp	1301 ± 16	1117 ± 17[b]	865 ± 13[b]	687 ± 34[b]	4289 ± 131

Note. Plasma concentrations corresponded to a 50% probability of no response (EC_{50}) to a stimulus. GHB and ethanol were administered alone (control groups), and GHB was also administered when increasing steady-state concentrations of ethanol (1000, 2000, and 3000 μg/ml) were targeted. EC_{50G} and EC_{50E} are the EC_{50} of GHB and ethanol, respectively, when administered alone, and $EC_{50,\ APP}$ is the apparent EC_{50} of GHB in the presence of a certain steady-state concentration of ethanol.

[a] The estimation proved impossible as the administration of ethanol alone already induced a loss of the whisker reflex in more than 70% of the cases. Results are expressed as mean ± SEM.

[b] $p < 0.05$ compared with the EC_{50G} by means of a Student *t*-test.

midazolam (Bol *et al*., 2000). GHB and ethanol, when administered alone, also differed in the sequence of the loss of the stimulus-response measures. GHB induced a loss of the startle reflex before the loss of the righting reflex, which is in agreement with our previous results (Van Sassenbroeck *et al*., 2002a). During the administration of ethanol, the righting reflex was lost first, followed by the startle reflex. The latter sequence is comparable with dexmedetomidine, midazolam, and thiopental (Bol *et al*., 2000; Gustafsson *et al*., 1996). The plasma concentrations at loss or return of the righting reflex measured in our study are in agreement with previously reported values for ethanol (Hisaoka and Levy, 1985a,b) and GHB concentrations (Van Sassenbroeck *et al*., 2001). Data for GHB and ethanol concentrations at disappearance of the reaction to a painful stimulus are not available in the literature.

The interaction between GHB and ethanol on the disappearance of some reflexes was studied next. With regard to the righting reflex, the $EC_{50,\ APP}$ of GHB decreased with increasing concentrations of ethanol, and the interaction factor *S* indicates that a synergy occurred with higher ethanol concentrations (>2 mg/ml). At lower ethanol concentrations, however, synergy with GHB was not observed, which illustrates that the type of interaction may be concentration dependent (Berenbaum, 1989). For the interaction between flurazepam and ethanol in the rat, the same pattern with additivity at low ethanol concentrations and synergism at higher ethanol concentrations was observed (Hu *et al*., 1986).

For the tail clamp reaction, the interaction appeared to be slightly antagonistic, which has also been reported for the interaction between isoflurane and ethanol (Johnstone *et al*., 1975), while for propofol and ethanol it was additive (Garfield and Bukusoglu, 1996). A concentration-dependent interaction was also observed for the startle reflex. At low ethanol concentrations, the steady state GHB concentration needed to induce a 50% loss was indeed significantly larger than in the control group receiving GHB alone (group 4) (Table 2). This was reflected in the shift to the right of the effect versus GHB steady-state concentration curve (Fig. 2). The synergy factor *S* was negative, and significantly different from zero, indicating antagonism. When higher concentrations of ethanol were targeted, additivity was observed with an *S* value which was not statistically significant different from zero. One explanation for the biphasic effect, with antagonism at low ethanol concentrations and additivity or synergism at higher concentrations, might be the biphasic effect of ethanol with low concentrations causing an activation of the brain and higher concentrations inducing neurological depression (Rossetti *et al.*, 2002). However, our experiments do not allow us to draw firm conclusions whether GHB differently modifies the effects of the different doses of ethanol. The differences in nature and extent of the interaction observed in our experiments for the different stimulus-response measures illustrate that the use of several endpoints is necessary to characterize the depth of sedation, hypnosis, and analgesia as measures for the "depth of anaesthesia" (Gustafsson *et al*., 1996; Hu *et al*., 1986) and corroborate the concept that various effects of anaesthetics are affected through different pathways in the central nervous system (Kissin *et al*., 1987; Reinoso-Barbero and de Andrés, 1995).

Finally, it should be mentioned that the values obtained for the interaction parameter *S* in our experiments with GHB and ethanol are relatively low compared with those reported for other interactions, e.g., the dexmedetomidine-midazolam interaction where *S* values from 7.3 for the whisker reflex up to 374 for the tail clamp reaction have been calculated (Bol *et al*., 2000). A possible explanation may be that GHB and ethanol share a common pathway for the induction of hypnosis, which may be GABA-ergic (Carai *et al*., 2001) or via the inhibition of dopamine release (Roth and Suhr, 1997). It has indeed been shown that the interaction between two drugs will be less effective if they induce the observed effect via a shared common pathway (Berenbaum, 1989; Bol *et al*., 2000; Garfield and Bukusoglu, 1996).

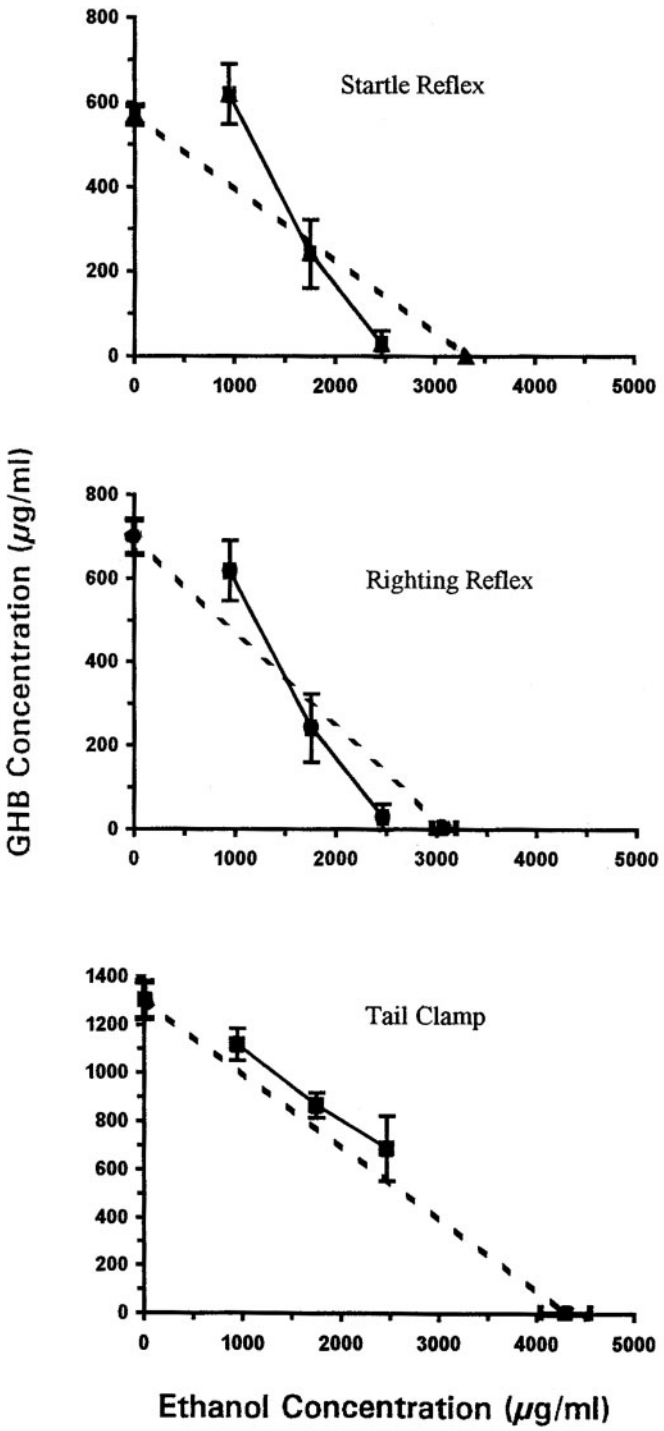

FIG. 3. $EC_{50,\ APP}$ isobolograms for the startle reflex, the righting reflex, and the tail clamp reaction. The $EC_{50,\ APP}$ values were estimated with Equation 5. The dashed straight line represents the curve when purely additive interaction will occur ($S = 0$). The degree of deviation from the line of additivity corresponds to the degree of synergy ($S > 0$) or antagonism ($S < 0$) of the interaction. Each point represents the estimated $EC_{50,\ APP}$ value with its 95% confidence interval.

It is concluded that our data confirm that ethanol prolongs the sleeping time of GHB. This is not likely to be explained by a pharmacokinetic interaction but is probably due to a pharmacodynamic action. A synergistic action is only observed with higher ethanol concentrations for the righting reflex. For the startle reflex, the interaction was antagonistic with lower ethanol concentrations and only additive at higher concentrations. With the tail clamp reaction, only a slight antagonism was observed. These findings underline the necessity of studying different stimulus-response measures at different concentrations to elucidate the action of and the interaction between hypnotics.

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