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Diazepam–Morphine Hypnotic Synergism in Rats

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The effect of diazepam-morphine combination on the righting reflex was studied in rats. Doses of the drugs given alone and in combination that block righting reflex (RR ED_{50}) were determined with a probit procedure. Brain concentrations following equieffective doses of the drugs administered separately and in combination were determined by radioimmunoassay. Equieffective intravenous doses and corresponding brain concentrations for the agents were compared with fractional (algebraic) and isobolographic analyses. Interaction between diazepam and morphine was found to be synergistic. It is not likely to be pharmacokinetic in nature. (Key words: Analgesics: morphine. Anesthetics, intravenous: diazepam. Interactions (drug), synergism: opioids; benzodiazepines. Pharmacodynamics: depth of anesthesia. Pharmacokinetics: brain concentrations of drugs.)

OPIOIDS ARE KNOWN to enhance the hypnotic effect of benzodiazepines in surgical patients.[1–4] It has been found also that naloxone antagonizes diazepam-induced hypnosis.[5] Although benzodiazepine and opioid receptors are pharmacologically separate, there are suggestions that these receptor systems may interact synergistically in the mediation of their effects.[6] The aim of the present study was to define the type of diazepam-morphine hypnotic interaction and also to determine whether pharmacokinetic factors play an important role in this interaction. The latter question is especially pertinent because the wide interindividual variability in response to benzodiazepines[7,8] has been attributed to pharmacokinetic factors[7] and because opioids have been found to decrease this variability.[2]

Materials and Methods

Experiments were performed on male Sprague-Dawley rats weighing 225–275 g. The protocol for this study was approved by the Institutional Panel on Laboratory Animal Care. As an endpoint for hypnotic effect, we used loss of the righting reflex, regarding the righting reflex test as positive if the rat failed to right itself (with all four feet on the table) within 15 s after being placed on its side. The observer was blinded to the dose of a drug. The experiments were carried out with the rats in a clear chamber, 30 × 25 × 40 cm, into which oxygen was delivered (4 l/min). The rat's hind leg (for injection into the saphenous vein) could be extended outside the chamber through a slot. The animals were placed in the chamber with oxygen at least 15 min before a first injection. Each animal was given one predetermined dose of diazepam, morphine, or diazepam–morphine combination. Times between injections of drugs and the righting reflex test were based on the times to peak effect for these agents: 15 min for morphine and 3 min for diazepam. The peak times were chosen after preliminary experiments in which the onset of loss of the righting reflex following injection of either diazepam or morphine was determined in a group of rats. The minimal time needed for loss of the righting reflex in all animals of a group (6–7 rats) was used for this. In the combined drug experiments, each drug was injected separately, so as to synchronize the occurrence of the peak effects. All experiments were carried out between 8:00 A.M. and 12 noon.

The study included three series of experiments: 1) a series for dose-related interaction, 2) a series for brain-concentration-related interaction, and 3) a series with Pa_{CO_2} measurements. In the first series of experiments, the interaction between diazepam and morphine was determined in two steps.[9] First, dose-effect curves were obtained and ED_{50} values calculated. Second, isobolographic and algebraic (fractional) analyses were used to characterize the type and degree of the interaction. In the first step, three dose-effect curves (three subseries of experiments) were determined: two with diazepam and morphine given alone and a third subseries with a diazepam-morphine combination. Five (or four) groups of four animals were used to determine the curve in each subseries of experiments, with doses equally spread to give a range of doses that block the righting reflex in none or all of the animals in a group. The diazepam-morphine weight ratio in the combined subseries was 1:4. This ratio was based on the data obtained in preliminary experiments on the relative hypnotic potencies of diazepam and morphine. From these experiments, it was found that diazepam was approximately four times as potent as morphine regarding loss of the righting reflex. As a result, we maintained the diazepam–morphine ratio at approximately equipotent level. The doses of both components of the combination rose by steps from one group of rats to an-

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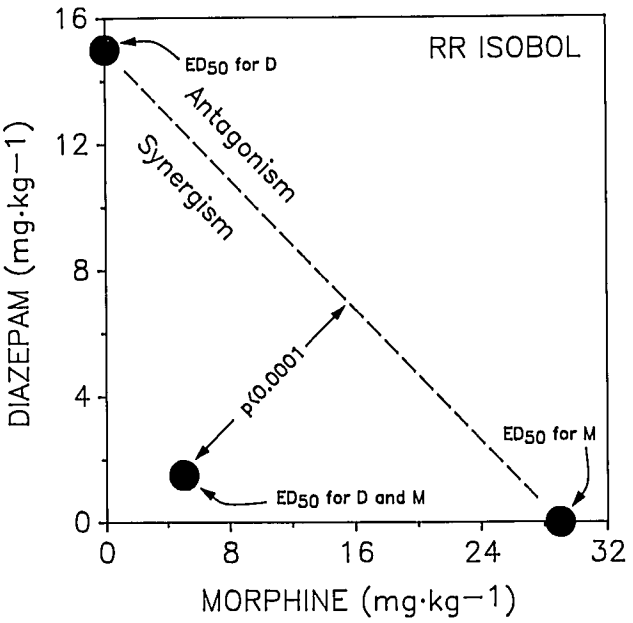

FIG. 1. ED_{50} isobologram for the interaction of diazepam and morphine regarding loss of the righting reflex (RR). The dashed straight line connecting the single-drug ED_{50} points is the additive line. *P* value indicates the level of statistical significance for deviation of the combined ED_{50} point from the additive line.

other with the constant weight ratio between the components. The following doses of the agents were used. In the diazepam subseries, the doses were 4, 8, 15, and 25 $mg \cdot kg^{-1}$. In the morphine subseries, the doses were 15, 20, 25, 30, and 40 $mg \cdot kg^{-1}$. In the diazepam–morphine subseries, the doses were 0.75 and 3.0, 1.0 and 4.0, 1.25 and 5.0, 1.5 and 6.0, and 2.0 and 8.0 $mg \cdot kg^{-1}$ (diazepam and morphine, respectively). The construction of the dose-effect curves and determination of ED_{50} values were based on the probit procedure.[10] The ED_{50} values were used for isobolographic[11] and algebraic (fractional)[12] analyses.

Isobolographic analysis allows one to visualize the nature of the interaction. An isobol is a line on a dose-dose surface denoting dose combinations that elicit the same magnitude of response.[13,14] ED_{50} values from all three subseries of experiments were plotted in the form of an isobologram (fig. 1), with single-drug ED_{50} points placed on the dose coordinates of the isobologram and a combined ED_{50} point in the dose-field. The deviation of a combined ED_{50} point from an additive line (straight line joining single-drug ED_{50} points) was measured as the length of a line running from the point in question to the additive line in a perpendicular manner. This distance was used to determine whether a statistically significant difference was present. The standard error of this distance was computed by the method of propagation of error,** and error estimates from a combined ED_{50} point, as well as single-drug ED_{50} points, were used. An approximate *t* test used to test the assumption of additivity was then obtained as the ratio of the measured distance to its standard error.[15]

Algebraic (fractional) analysis was based on the expression of doses of the diazepam and morphine components of the combination (Dc and Mc) as fractions of the doses of these drugs that produce the same effect when given separately (Dc/Ds and Mc/Ms). The sum of fractional doses equals to 1.0 in summation, as expressed by the following equation:

$$\frac{Dc}{Ds} + \frac{Mc}{Ms} = 1.0$$

In synergism, the sum of fractional doses is less than 1.0, and in antagonism, it is more than 1.0 (table 1).[12] Statistical validation for fractional (algebraic) analysis duplicated that for isobolographic analysis.

In the second series of experiments, the interaction between diazepam and morphine was studied in relation to brain concentrations of these drugs. In this series, brain concentrations of diazepam and morphine were determined at the peak of the hypnotic action of these agents administered intravenously at the ED_{50} level (determined in the first series of experiments). Three groups of animals were used in this series: 1) diazepam, 2) morphine, and 3) diazepam–morphine group (table 2). Animals were decapitated 3 min after injection of diazepam in the first group, 15 min after injection of morphine in the second group, and 15 or 3 min after injection of morphine and diazepam (respectively) in the third group. The brain was immediately excised, freed of blood vessels and choroid plexa as much as possible, weighed, and refrigerated. After addition of 0.5 ml 70% $HClO_4$, the whole brain was homogenized for 30 s with a Polytron Homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at 3000 × g for 30 min. Fifteen hundredths ml of 50% NaOH was added to the supernatant and it was centrifuged for another 10 min to produce a clear supernatant. The supernatant was assayed for morphine (in the first and third groups) and diazepam (in the second and third groups) by radioimmunoassay with ^{125}I-labelled morphine and ^{125}I-labelled oxazepam (Kit Abuscreen®, Roche Diagnostis Systems, Hoffman-La Roche, Inc., Nutley, NJ).

Both radioimmunoassays are based upon the competitive binding to antibody of ^{125}I radiolabeled antigen and unlabeled antigen, in proportion to their concentrations in the reaction mixture.[16–18] An unknown sample (brain tissue supernatant) was mixed in a test tube with fixed amounts of anti-morphine (or anti-benzodiazepine) serum and radiolabeled antigen. Antigen present in the sample competes with labeled antigen for the limited antibody

** Ku HH: Notes on the use of propagation of error formulas. J Res Natl Bureau Stand 70:263–273, 1966

TABLE 1. Dose-related Diazepam–Morphine Hypnotic Interaction

Subseries	Equieffective IV Doses (ED_{50}) $\left(\frac{\text{fraction}}{\text{mg/kg}}\right)$		Sum of Fractions	Ratio†
	Diazepam Component	Morphine Component		
Diazepam (n = 16)	$\frac{1.00}{14.9\ (8.8,\ 25.1)}$*	0.00	1.00	—
Morphine (n = 20)	0.00	$\frac{1.00}{28.5\ (21.7,\ 37.3)}$	1.00	—
Diazepam and Morphine (n = 20)	$\frac{0.08}{1.2\ (1.0,\ 1.4)}$	$\frac{0.16}{4.7\ (4.0,\ 5.6)}$	0.24 $P < 0.0001$	4.2

* Fiducial limits in parentheses.
† Ratio of single drug fractional dose to combined fractional dose. The P value denotes the significance of the difference between combined fractional dose and single-drug fractional dose.

present. After precipitation of the antigen-antibody complex with a second antibody reagent and centrifugation, the tubes were decanted, drained, blotted, and the pellets containing bound antigen were counted in a gamma scintillation counter calibrated for ^{125}I. For calculations, standard curves were prepared by plotting percent bound *versus* morphine (or diazepam) concentration. A best-fit curve for this relationship was obtained by using "RIA Data Reduction" computer program (American Society of Clinical Pathologists). Sample concentrations were obtained by interpolating from the standard curve.

Abuscreen test kit for morphine consists of anti-morphine serum reagent (goat), ^{125}I-morphine reagent, positive reference standard (morphine sulfate, 300 ng/ml), normal reference standard, and second antibody reagent (donkey). The sensitivity of the radioimmunoassay for morphine is 10 ng/ml, with the variability of the assay ±10%, and cross reactivity with the major morphine metabolite, morphine-3-glucuronide 38%. Morphine glucuronides are more polar- and less lipid-soluble than unchanged morphine and, therefore, have very slow rate of diffusion into cerebrospinal fluid.[17,19] We took brain samples 15 min after morphine injection. Under such conditions, low specificity of the morphine assay (regarding the major metabolite) was of little importance, because it did not have enough time to be accumulated in the brain. The assay has been found not to cross-react with diazepam.

Abuscreen test kit for benzodiazepines consists of anti-benzodiazepine serum reagent (sheep), ^{125}I-oxazepam derivative, positive reference standard (oxazepam, 100 ng/ml), normal reference standard, and second antibody reagent (donkey). The sensitivity of the benzodiazepine radioimmunoassay is 5 ng/ml with high cross-reactivity with diazepam desmethylated metabolites. However, the problem of cross-reactivity with the metabolites was not important for our study because, 3 min after intravenous injection of diazepam, brain level of desmethylated metabolites is undetectable.[20] The assay has been found not to cross-react with morphine. The recovery of the added diazepam and morphine averaged 96% and 93%, respectively.

Brain concentrations were used for assessment of drug interaction with fractional analysis. For this, the brain concentrations of morphine and diazepam in the combined group of experiments (third group) were expressed as fractions of the concentrations of these agents administered alone (first and second groups, table 2) a technique similar to that used with the dose-related fractional (algebraic) analysis. The sum of the diazepam and morphine fractions was compared with 1.0 to determine the direction (type) and degree of interaction.

In the third series of experiments, the effects of morphine and morphine–diazepam combination on Pa_{CO_2} were studied. This series of experiments included prior preparation of the rats by insertion of a catheter (PE-10) *via* the femoral artery into the aorta, with the peripheral end of the catheter tunneled subcutaneously and exteriorized at the back of the neck. Heparinized saline (100

TABLE 2. Brain Concentration-related Diazepam–Morphine Hypnotic Interaction

Groups with Doses at ED_{50} Level	Equieffective Brain Concentrations $\left(\frac{\text{fraction}}{\text{ng/g}}\right)$		Sum of Fractions	Ratio†
	Diazepam Component	Morphine Component		
Diazepam 15 mg/kg (n = 5)	$\frac{1.00}{718 \pm 153}$*	0.00	1.00	—
Morphine 28.5 mg/kg (n = 5)	0.00	$\frac{1.00}{714 \pm 75}$	1.00	—
Diazepam 1.2 mg/kg and Morphine 4.7 mg/kg (n = 4)	$\frac{0.10}{75 \pm 17}$	$\frac{0.15}{105 \pm 6}$	0.25 $P < 0.0001$	4.0

* Standard deviation.
† Ratio of single drug brain concentration fraction to the sum of fractions in combination. The P value denotes the significance of the difference of the sum of fractions from 1.00.

TABLE 3. Interaction of Morphine with Intravenous Nonnarcotic Anesthetics in Relation to Hypnotic Effect in Rats

Endpoint of Anesthesia	Combinations at ED_{50} Level		
	Diazepam + Morphine	Thiopental + Morphine[19]	Etomidate + Morphine[20]
Loss of the righting reflex	R = 4.2*	R = 2.3*	R = 1.6*

R = Ratio of single-drug fractional dose to combined fractional dose reflecting the degree of synergism (R > 1.0).
* Significance from the additive effect at $P < 0.001$.

U/ml) maintained patency of the catheter. On the day of the experiment, the rats received intravenous injections of morphine or morphine–diazepam in equipotent doses (ED_{50} for loss of the righting reflex) determined in the first series of experiments. Blood samples (0.2 ml) were withdrawn from the catheter before the injections and 3 min after the second injection (diazepam). Immediately before taking a sample, the investigator withdrew 0.15 ml of blood from the catheter to prevent 0.2 ml dead space from affecting the Pa_{CO_2} measurement (the 0.15 ml was injected back after the sample was taken). Arterial blood gas tensions were measured using an IL System 1303 Blood Gas Analyzer® (Instrumentation Laboratory, Inc.). For comparisons between means of the groups to which morphine and morphine–diazepam were administered, a two sample *t* test was used. For changes within a group, a paired *t* test was used.[21]

Morphine sulfate used in the study was purchased from Eli Lilly (Indianapolis, IN), and diazepam was a gift from Hoffmann La Roche (Nutley, NJ). Doses of morphine were expressed in terms of the salt. Morphine was dissolved in isotonic saline, and diazepam in a propylene glycol (80%)-ethanol (20%) solvent. When diazepam was given in combination with morphine, the solvent was diluted ten times in isotonic saline (maximal dilution without precipitation of diazepam). This was done to prevent possible effect of the solvent for diazepam on the morphine hypnotic action. In a pilot series of experiments, we studied the effect of the diluted solvent (0.4 ml) on the hypnotic dose-response curve for morphine and found no change in the position of the curve along the dose axis. Total volume of injections was 0.5–0.8 ml. Morphine was injected over 15 s, and diazepam over 60 s.

Results

The dose-related diazepam-morphine isobologram for loss of the righting reflex is presented in figure 1. The combined ED_{50} point deviates ($P < 0.0001$) from the additive line (joining single-drug ED_{50} points), indicating synergism. Fractional (algebraic) analysis of this interaction is presented in table 1. In combination, the sum of fractional doses was significantly lower than a single-drug fractional dose (0.24 *vs.* 1.00, $P < 0.0001$). As a result, the ratio of a single-drug fractional dose to the combined fractional dose indicated a degree of synergism of 4.2.

Table 2 reflects the results of the second series of experiments. When brain concentrations of diazepam and morphine given in combination (at ED_{50} level) were expressed as fractions of the concentrations of these agents administered alone (at the equieffective doses), the outcome did not differ from that for the dose-related interaction. In combination, the sum of fractional concentrations was significantly less ($P < 0.0001$) than the fractional concentration of a single agent. The degree of synergism reflected by the ratio of a single-drug fractional concentration to the combined fractional concentration was almost the same as with dose-related analysis of the interaction (4.0 *vs.* 4.2).

In an attempt to evaluate the possible role of ventilatory depression (hypercarbia) in outcome of the diazepam-morphine hypnotic interaction, we performed the Pa_{CO_2} experiments. At the ED_{50} dose level, morphine caused an increase in Pa_{CO_2}, from 33.0 ± 1.9 mmHg to 63.5 ± 11.5 mmHg ($P < 0.001$), whereas the administration of diazepam-morphine combination in the equieffective dose resulted in a Pa_{CO_2} change from 29.5 ± 1.9 mmHg to 36.1 ± 1.9 mmHg ($P < 0.001$). Thus, the combination caused a less pronounced change in Pa_{CO_2} than that caused by morphine alone (6.6 ± 1.7 mmHg *vs.* 30.5 ± 9.9 mmHg, $P < 0.001$).

Discussion

The algebraic (fractional) and isobolographic analyses used in the present study demonstrated synergistic morphine-diazepam interaction in relation to the hypnotic effect. Several groups of authors[1–3] reported strengthening the hypnotic effect of benzodiazepines with opioids in surgical patients. An increase in the effect of alfentanil by diazepam was also reported.[22] However, these studies did not indicate whether the observed results reflected synergism or simple summation of the effects. Use of the ratio of single-drug fractional dose to combined fractional dose in the present study gave the opportunity to measure the degree of synergism (R > 1.00), which reached an R = 4.20 level with the morphine-diazepam combination. We have found previously in similar experiments that combinations of morphine with thiopental or etomidate also result in a synergism with regard to the hypnotic effect[23,24]; however, the synergism between morphine and diazepam seems the most pronounced (table 3).

Combined drug administration may result not only in pharmacodynamic, but also in pharmacokinetic, interactions with appropriate changes in the concentrations of interacting agents at their sites of action. The brain-con-

centration variables characterize brain sensitivity to the drugs and therefore are most likely independent of pharmacokinetic considerations. However, one should take into account that samples from the whole brain may not exactly represent drug concentrations at a specific brain target site. Our results with brain-concentration-related analysis of the morphine–diazepam interaction demonstrated the same degree of synergism as results with dose-related analysis of the interaction showed. This outcome decreases probability that pharmacokinetic factors play any significant role in the morphine–diazepam hypnotic synergism. It is of interest here that age-related changes in patient sensitivity to opioids (fentanyl, alfentanil)[25] and to midazolam[26] have been found recently to be independent of pharmacokinetic factors.

The synergistic interaction between morphine and diazepam cannot be explained on the basis of their interaction at a common receptor complex. Benzodiazepine and opioid receptors are pharmacologically separate. It has been reported that morphine does not affect benzodiazepine receptors directly, as evidenced by the lack of changes in the affinity and density of the receptors.[27] At the same time, morphine has an effect on the GABA receptor-ionophore complex where the benzodiazepine receptor is located. It has been found that morphine blocks the GABA-A receptor for which it has only moderate affinity.[28–30] Thus, morphine in the CNS acts at two separate receptor sites: opioid receptors, which it activates at low doses (naloxone-reversible effect); and GABA-A receptors, which it blocks at relatively high doses (nonnaloxone reversible effect). The interaction of benzodiazepines and opioids at the GABA receptor-ionophore complex could potentially lead to antagonism between diazepam (facilitating the action of GABA) and morphine (blocking the GABA receptor). But this mechanism can hardly be used for explanation of the diazepam-morphine hypnotic synergism obtained in our experiments. We suggest that there is a functional link between the GABA receptor-benzodiazepine receptor system and the opioid receptor system in the mediation of hypnosis. Stella *et al.*[5] have reported that, in surgical patients, naloxone decreased the percent of patients rendered unconscious by diazepam. Because naloxone was used in a small dose (6 $\mu g \cdot kg^{-1}$), nonspecific, analeptic effect of this agent can probably be ruled out. This finding may suggest that an endogenous opioid system provides some complementary action for benzodiazepine-induced hypnosis. Thus, most likely morphine-diazepam hypnotic synergism represents a functional interaction, when two different systems are complimentary for the common effect.

The depressant effect of the morphine-diazepam combination on ventilation, resulting in hypercarbia, might contribute to the hypnotic effect; however, the Pa_{CO_2} measurements showed that, at the equieffective dose level (hypnotic ED_{50}), the morphine–diazepam combination caused an increase in Pa_{CO_2} to a lesser extent than that caused by morphine alone. The small increase in Pa_{CO_2} with the morphine–diazepam combination (6.6 ± 1.7 mmHg) probably relates to the fact that, at the hypnotic peak effect (3 min after the injection of diazepam), the accumulation of CO_2 is not yet maximal (corresponding to a new ventilatory steady state). Increasing Pa_{CO_2} up to 95 mmHg does not affect halothane MAC,[31] and only a Pa_{CO_2} of 245 mmHg reduces halothane MAC to zero[32]; therefore, the increase in Pa_{CO_2} in the present study was too small to contribute to the hypnotic synergism of the morphine–diazepam combination.

In conclusion, the interaction between diazepam and morphine in rats was found to be synergistic. It is not likely to be pharmacokinetic in nature.

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