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Isobolographic Analysis of the Interaction Between Epidural Sufentanil and Bupivacaine in Rats

MARCEL VERCAUTEREN* AND THEO F. MEERT†

*Department of Anesthesiology, University Hospital Antwerp, B-2650 Edegem, Belgium
†Department of Neuropsychopharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium

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VERCAUTEREN, M. AND T. F. MEERT. *Isobolographic analysis of the interaction between epidural sufentanil and bupivacaine in rats.* PHARMACOL BIOCHEM BEHAV **58**(1) 237–242, 1997.—The present study was performed to evaluate the nature of the interaction between epidurally administered sufentanil and bupivacaine in producing antinociception in rats. Rats in which epidural catheters had been inserted received epidural injections with bupivacaine and sufentanil. Nociception was tested by use of the tail-withdrawal reaction (TWR) test and the hot-plate test. Isobolographic analyses were performed with fixed and variable dose ratio treatment schedules based on the ED_{50}s and the highest inactive concentrations of the compounds in both tests. In the TWR test, a synergistic interaction was obtained between the two compounds independent of whether a variable dose ratio regimen (with either 0.08 μg/rat sufentanil or 80 μg/rat bupivacaine as the preset component) or a fixed dose ratio of 1/1,000 sufentanil/bupivacaine (based on the individual ED_{50}s) was used. In the hot-plate test, a synergistic interaction was observed only in the variable dose ratio regimen with 0.08 μg/rat sufentanil as the preset component and in the fixed dose ratio regimen of 1/1,000 sufentanil/bupivacaine (a ratio based on the ED_{50} values of the TWR test) but not with a ratio of 1/200, as demonstrated by the ED_{50}s of both drugs in the hot-plate test. The interaction between epidurally administered bupivacaine and sufentanil seems to be synergistic for both tests when variable and fixed dose ratios are used. The synergism could be more easily demonstrated in the TWR test. For drugs with a segmental action, the hot-plate test seems to be less optimal. The necessity of a minimal critical amount of bupivacaine to obtain synergism may have clinical implications. © 1997 Elsevier Science Inc.

Bupivacaine Sufentanil Epidural administration Isobolographic analysis Variable dose ratio Fixed dose ratio

PREVIOUS work on the potentiating effect between local anesthetics and opioids was limited to the study of interactions between lidocaine or bupivacaine and the hydrophilic opioid morphine after intrathecal administration (1,11,16). In a preceding study (23), using epidural administration of bupivacaine and the lipophilic opioid sufentanil in rats, we suggested a potentiation between the two agents in the tail-withdrawal reaction (TWR) test.

To better characterize the interaction between bupivacaine and sufentanil, a new series of experiments was conducted. These additional studies were designed to determine the nature of the interaction between the two agents by use of fixed and variable dose ratio concentration regimens. In a fixed dose ratio regimen, a fixed ratio between the two selected compounds is always used for the testing of the different drug mixtures. The selection of the ratio between the two compounds is based on the potency (e.g., ED_{50}s) of the compounds under the experimental conditions used. The fixed dose ratio procedure allows calculation of the ED_{50}s of additivity, which can be compared more easily with the measured values, while respecting the confidence limits of both components (10,18,22). In the variable dose ratio method, one drug dose is held constant and various doses of the second compound are added. As a result, the drug ratios between the two compounds differ for each drug mixture tested (6,14,15,18, 19,25). Several studies have used the variable dose ratio method, and several reasons have been stressed as to why this method is less suitable for an isobolographic analysis than is

Requests for reprints should be addressed to Theo F. Meert, Janssen Research Foundation, Department of Neuropsychopharmacology, Turnhoutseweg 30, B-2340 Beerse, Belgium. E-mail: tmeert@janbe.jnj.com

the fixed dose ratio method. First, in the combination, the drug with the preset and thus constant dose does not have any confidence limits. Second, the ED_{50} with confidence limits of the second drug has to be compared with a value on the theoretical line of additivity corresponding with a completely different dose ratio regimen. For this reason, the fixed dose ratio method seems to be more appropriate, enabling statistical analysis by Student's *t*-test to compare the differences between the experimental and theoretical points of additivity, both situated on the diagonal fixed dose ratio line (17,18). The selection of the fixed dose ratio may, however, be a problem, especially if the real relative potency cannot be estimated because of the inability to calculate the exact ED_{50}s of one or both compounds (13). Therefore, both the fixed and the variable dose ratio methods sometimes need to be used.

The present study differs from our previously published work in that both a fixed dose ratio regimen and a two-directional variable drug concentration ratio were used. Furthermore, two behavioral assays (the tail-withdrawal reaction test and the hot-plate test) were used in order to evaluate whether different behavioral tests may result in different outcomes. Whereas the tail withdrawal reaction test is based on a spinally mediated response, responses in the hot-plate test involve supraspinal components.

MATERIALS AND METHODS

Animals

Approval from the Institutional Animal Care and Use Committee was obtained to perform the experiments described. Male Wistar rats ($n = 145$) weighing 250 ± 20 g were used for epidural catheterization according to a technique described in detail elsewhere (12,13). With the animals under general anesthesia, a polyethylene catheter (PE10) was introduced into the epidural space over a length of 0.5 cm cephalad via a hole drilled in the fourth lumbar vertebra. Upon fixation of the catheter to the vertebra, the free end was tunneled subcutaneously toward the occiput. The animals were allowed 4 days to recover from anesthesia and surgery. During this time they had free access to food and water. Animals showing any sign of apparent neurological damage were discarded. After the experiments, in which the animals were used only once, the rats were killed and the position of the catheter tip was checked at autopsy by an experienced investigator who was blind to the results. Only the results from those animals with catheter tips located in the epidural space, and without any sign of fibrinous tissue reaction around the catheter, were used for data analysis. All experiments and housing after surgery took place in an air-conditioned laboratory (temperature 21 ± 1°C, humidity 65 ± 10%).

Experimental Design

The animals were assigned at random to receive an epidural injection of sufentanil (0.08, 0.16, 0.31, 0.63, 1.25, 2.5, or 5.0 μg per rat), bupivacaine (40, 80, 160, 320, or 640 μg/rat), a combination of 80 μg of bupivacaine with variable doses of sufentanil, a combination of 0.08 μg sufentanil with variable doses of bupivacaine, or fixed doses representing 1/1,000 and 1/200 concentration ratios of sufentanil to bupivacaine (based on the individual ED_{50}s of sufentanil and bupivacaine obtained first in the TWR test and the hot-plate test, respectively). All drug injections were given in a single treatment volume of 10 μl. Epidural doses were given on a micrograms/rat basis rather than a micrograms/kilogram basis. The former regimen is also more customary in humans. Both bupivacaine and sufentanil citrate were prepared fresh as aqueous solutions. The desired volume was administered in consecutive steps of 1 μl with care taken that the dead space of the catheter was filled with saline, separated from the injectate by 3 μl of air.

Data were collected from five animals per drug treatment condition by a single observer unaware of the pharmacological treatment. TWR latency was scored once before and at 15, 30, and 60 min after the injection. The hot-plate test was performed twice before ($t - 15$ min and $t - 5$ min) and at 20, 40, and 70 min after injection. To reduce the number of animals to be used, both tests were performed in the same animals.

Tail-Withdrawal Reaction Procedure

The TWR used here has been described in detail by Janssen et al. (7). The rat was placed in a cylindrical rat holder with its tail hanging freely outside the cage. The distal 5 cm of the tail was immersed in a warm water bath (55 ± 1°C), and the time for tail withdrawal was measured to the nearest 0.1 s. To minimize tissue damage on repeated testing, a cutoff time of 10.0 s was adopted.

Hot-Plate Test

The hot-plate test was performed with a plate at 55 ± 1°C. The latency was determined as the time between the animals being placed on the surface and the registration of either vocalization, licking of the hindpaws, or jumping. A cutoff time of 30.0 s was used.

Data Analysis

Criterion values were defined for each of the two behavioral assays examined. The TWR latency was evaluated by use of criteria of >6.0 s and $\geqslant 10.0$ s, representing validated moderate and strong antinociceptive effects (7). Two criterion values were included in the TWR test to determine whether the magnitude of stimulation would result in different outcomes within one test procedure. For the hot-plate test, a cutoff latency of $\geqslant 30.0$ s was applied.

All data were analyzed in terms of the number of animals in each treatment condition that met the criterion. ED_{50} values and 95% confidence limits were calculated according to Finney's iterative method (5). For the theoretical calculations of ED_{50}s and confidence limits for the variable dose ratio concentration regimen, the exact values obtained on the intersection point with the line of theoretical additivity and their limits were calculated by use of classical linear regression analysis ($x/a + y/b = 1$).

The theoretical point (ED_{50}) of additivity and the corresponding confidence limits in the fixed dose ratio concentration regimen were calculated according to the formula of Tallarida et al. (22):

$$ED_{50}\ \text{addit} = ED_{50}(\text{compound 1})/(p1 + Rp2)$$

where R = calculated ratio of measured ED_{50}s of compound 1/compound 2 for the particular observation, $p1$ = proportion of compound 1 in the mixture, and $p2$ = proportion of compound 2 in the mixture.

Statistical differences between ED_{50}s were analyzed with Student's *t*-test for independent samples on differences of log ED_{50}s (two-tailed). The standard errors of the log ED_{50}s were obtained from the 95% confidence limits (20).

TABLE 1

ED_{50}s FOR SUFENTANIL, BUPIVACAINE, AND MIXTURES OF SUFENTANIL AND BUPIVACAINE

Drug Condition	TWR Latency ≥10.0 s	HP Latency ≥30.0 s
Sufentanil	0.39 (0.26–0.58)	1.02 (0.76–1.38)
Bupivacaine	308 (246–402)	216 (165–283)
Sufentanil with 80 μg bupivacaine	0.11 (0.08–0.17)**	0.45 (0.28–0.72)*
Bupivacaine with 0.08 μg sufentanil	85.7 (53.0–138.0)**	113.1 (91.3–140.2)
Ratio 1/1,000		
Sufentanil	0.085 (0.063–0.12)***	0.11 (0.091–0.14)***
Bupivacaine	85.74 (63.28–116.18)***	113.14 (91.27–140.25)**
Ratio 1/200		
Sufentanil	0.32 (0.24–0.44)	0.49 (0.36–0.67)*
Bupivacaine	64.98 (47.96–88.05)***	98.49 (72.68–133.45)**

ED_{50}s and 95% confidence limits are shown for a tail-withdrawal reaction (TWR) latency ≥10 s and a hot-plate (HP) latency ≥30.0 s after epidural administration in rats. Statistical differences between the ED_{50}s in the mixtures and the ED_{50}s of the compounds alone were evaluated with Student's *t*-test for independent samples on differences of log ED_{50} (two-tailed); standard errors of the log ED_{50}s were obtained from the 95% confidence limits (20).

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

RESULTS

Single Component Data

The ED_{50}s measured for a TWR latency >6.0 s, a TWR latency ≥10.0 s, and a hot-plate latency ≥30.0 s were 0.22 (0.13–0.36), 0.39 (0.26–0.58), and 1.02 (0.76–1.38) μg/rat for sufentanil and 308 (236–402), 308 (246–402), and 216 (165–283) μg/rat for bupivacaine (Table 1). For sufentanil, the ED_{50} in the hot-plate test was greater ($p < 0.05$) than those observed in the TWR test when both TWR latency criteria (>6.0 s and ≥10.0 s) were used. For bupivacaine, the ED_{50} for a hot-plate latency ≥30.0 s was less ($p < 0.05$) than the ED_{50}s for TWR latencies of >6.0 s and ≥10.0 s. Based upon the lack of activity with 80 μg bupivacaine and 0.08 μg/rat sufentanil, both concentrations were selected as the preset doses in the variable dose ratio regimen. Because the data obtained for a TWR latency >6.0 s were always comparable to those for a TWR latency ≥10.0 s, only the latter will be reported further.

Variable Dose Ratio Testing

The use of a preset sufentanil dose of 0.08 μg/rat (which was 20% of the ED_{50} dose for a TWR latency ≥10.0 s) produced reductions in the ED_{50}s of bupivacaine for a TWR latency ≥10.0 s and a hot-plate latency ≥30.0 s of 72% and 48%, respectively (see Table 1). All these reductions were significantly different ($p < 0.05$) from the ED_{50}s of bupivacaine alone (Figs. 1, 2, left panels).

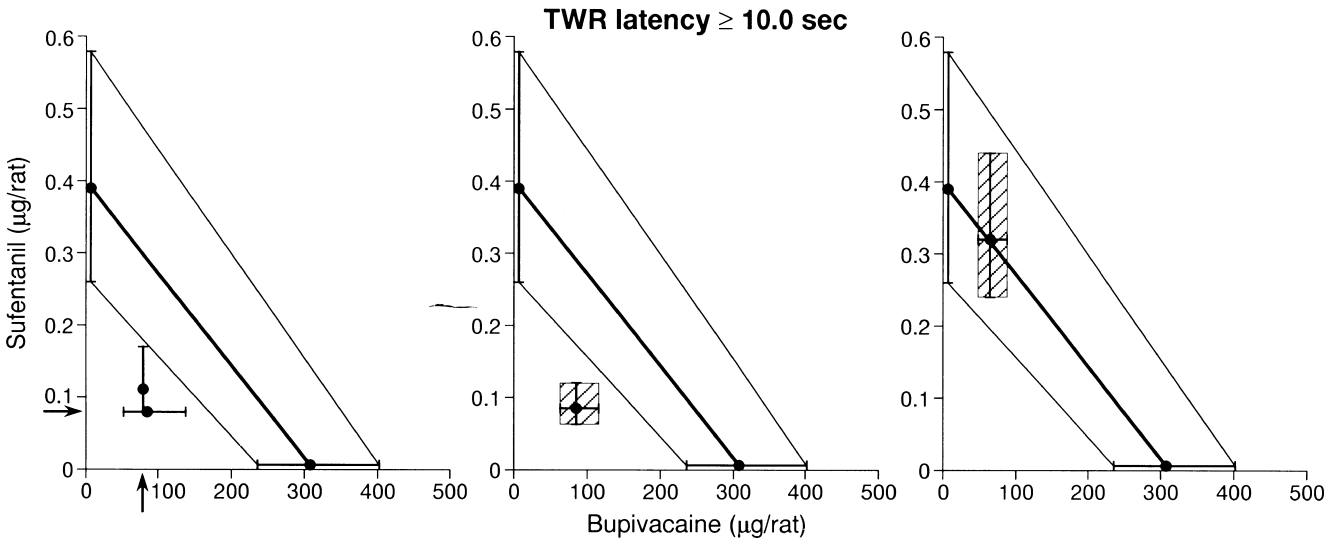

FIG. 1. Interactions between epidurally administered sufentanil and bupivacaine in the tail-withdrawal reaction test in rats. Presented are the ED_{50}s (and 95% confidence limits) of sufentanil and bupivacaine in a variable drug ratio regimen with fixed doses of 0.08 μg/rat sufentanil and 80 μg/rat bupivacaine (left panel), and in a fixed drug dose ratio of sufentanil/bupivacaine of 1/1,000 (based on the individual ED_{50}s of both compounds in the TWR test itself; middle panel) and 1/200 (based on the individual ED_{50}s of both compounds in the hot-plate test; right panel). For further information, see text.

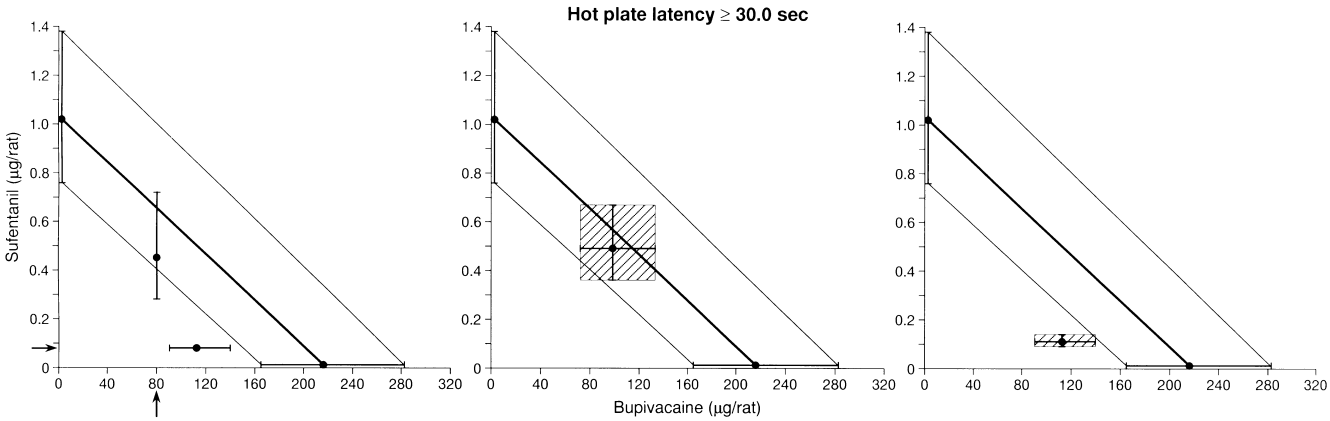

FIG. 2. Interactions between epidurally administered sufentanil and bupivacaine in the hot-plate test in rats. Presented are the ED_{50}s (and 95% confidence limits) of sufentanil and bupivacaine in a variable drug ratio regimen with fixed doses of 0.08 μg/rat sufentanil and 80 μg/rat bupivacaine (left panel), and in a fixed drug dose ratio of sufentanil/bupivacaine of 1/200 (based on the individual ED_{50}s of both compounds in the hot-plate test itself; middle panel) and 1/1,000 (based on the individual ED_{50}s of both compounds in the TWR test; right panel). For further information, see text.

The use of a preset bupivacaine dose of 80 μg/rat (which was 26% of the original ED_{50} for a TWR latency ⩾10.0 s) produced reductions in the ED_{50}s of sufentanil for a TWR latency ⩾10.0 s and a hot-plate latency ⩾30.0 s of 72% and 56% (see Table 1). Here also, all reductions were significantly different ($p < 0.05$) from the ED_{50}s of sufentanil alone (Figs. 1, 2, left panels).

Fixed Dose Ratio Regimen

Based upon the results obtained with both sufentanil and bupivacaine alone in the TWR test (i.e., a ratio of 1/1,400 for a TWR latency >6.0 s and a ratio of 1/790 for a TWR latency ⩾10.0 s), a fixed sufentanil/bupivacaine dose ratio of 1/1,000 was selected (Fig. 1, middle panel; Fig. 2, right panel). The ED_{50}s obtained with this fixed dose ratio regimen, using the all-or-none criteria for antinociception in the TWR and hot-plate test, were all lower ($p < 0.05$) than the corresponding ED_{50} values obtained with the single component data (Table 1). Within the ED_{50}s so obtained in the fixed dose ratio regimen of 1/1,000, sufentanil represented 22% and 11% of its original values; for bupivacaine, the corresponding values were 27% and 52%. Because with regard to the hot-plate testing the ED_{50} ratio of sufentanil/bupivacaine was 1/200, all tests were repeated with a fixed drug ratio regimen of sufentanil/bupivacaine of 1/200 (Fig. 1, right panel; Fig. 2, middle panel). The ED_{50} of sufentanil thus obtained in the TWR test was considerably higher than that obtained with the 1/1,000 ratio, and it was not statistically different from the ED_{50} of sufentanil alone. For the hot-plate test, a small statistical difference continued to exist between comparable ED_{50}s ($p < 0.05$). For bupivacaine, the ED_{50}s did not differ from those obtained with the 1/1,000 ratio. As a consequence, the difference from pure bupivacaine alone remained ($p < 0.05$).

Comparison Between Experimental and Theoretically Calculated Data

The various theoretically calculated ED_{50}s are presented in Table 2. The theoretical ED_{50}s of sufentanil with a fixed concentration of 80 μg bupivacaine for a TWR latency ⩾10.0 s and a hot-plate latency ⩾30.0 s were 0.29 and 0.64 μg/rat, respectively. Only for the TWR testing was the theoretical ED_{50} higher than that determined experimentally ($p < 0.05$). The

TABLE 2

CALCULATED ED_{50}s FOR SUFENTANIL AND BUPIVACAINE IN MIXTURES OF THE TWO DRUGS

Drug Condition	TWR Latency ⩾10.0 s	HP Latency ⩾30.0 s
Sufentanil with 80 μg bupivacaine	0.29 (0.17–0.47)*	0.642 (0.392–0.99)
Bupivacaine with 0.08 μg sufentanil	244.82 (163.38–346.55)**	199 (147.63–266.59)*
Ratio 1/1,000 (ED_{50} bupivacaine)	172.19 (123.77–237.57)*	178.39 (135.68–235.04)*
Ratio 1/200 (ED_{50} bupivacaine)	62.30 (42.65–90.13)	105.17 (79.31–140.07)

ED_{50}s and 95% confidence limits are shown for a tail-withdrawal reaction (TWR) latency ⩾10 s and a hot-plate (HP) latency ⩾30.0 s after epidural administration in rats. The ED_{50}s were calculated with either linear regression (in the case of the variable dose ratio regimen) or according to the formula for additivity of Tallarida et al. (22). Statistical differences between the calculated ED_{50}s and the corresponding ED_{50}s determined experimentally (Table 1) were evaluated with Student's *t*-test for independent samples on differences of log ED_{50} (two-tailed); standard errors of the log ED_{50}s were obtained from the 95% confidence limits (20).

*$p < 0.05$, **$p < 0.01$.

ED_{50}s of bupivacaine calculated for a fixed sufentanil dose of 0.08 μg/rat were in all cases higher than those actually measured. For the 1/1,000 ratio concentration, the calculated additive ED_{50}s were always higher than the experimentally determined ED_{50}s. Thus, the experimental results revealed stronger additive effects than what would be expected on theoretical grounds. For the 1/200 ratio, all differences between the theoretically calculated and the experimentally measured ED_{50} values disappeared ($p > 0.05$).

DISCUSSION

To analyze the possible interaction between two different agents in a particular assay, various approaches can be used. A technique often applied is the use of an isobolographic analysis, in which drug testing is performed with either a fixed or a variable dose ratio regimen (1,6,11,14,15,18,19,24,25). According to some, isoboles may not be the optimal method for demonstrating synergism when two drugs act on different receptor sites (3). However, when applying their proposed equations to our data, the present results of the TWR test are confirmed, and even for the hot-plate test synergy appears to exist for all the ratio methods and proportions of drug mixtures used. Therefore, isobolographic analysis remains a powerful technique for evaluating the nature of interaction between two compounds.

The selection of the opioid might be very important for interaction studies. Unlike previous studies (1,11,14–16,18), mostly using morphine and a local anesthetic, in the present study the lipophilic opioid sufentanil was used. Whereas morphine has a slower onset of action than bupivacaine, our previous experiments (23) have demonstrated similar time courses of onset, peak effect, and duration for sufentanil and bupivacaine. Due to the selection of sufentanil and bupivacaine, no major differences in redistribution of the compounds in the injected drug mixtures from the spinal space are expected, and the observed interactions will not be influenced by pharmacokinetic differences between the compounds.

Because the degree of antinociception may depend on the behavioral assay used (12), the interactions between sufentanil and bupivacaine were evaluated in two different tests for nociception: the TWR test and the hot-plate test. With each test, the compounds were evaluated alone, in combination with a preset but subactive dose of the other drug (variable dose ratio regimen), and in a fixed dose ratio regimen based on the relative potencies of the drugs in the two behavioral assays. Because the potency ratios based on ED_{50}s were 1/1,000 and 1/200 sufentanil/bupivacaine for the TWR test and the hot-plate test, respectively, fixed dose ratio testing was performed with sufentanil/bupivacaine concentrations of both 1/1,000 and 1/200 in both behavioral tests.

In the TWR test, a synergistic interaction was obtained between epidural sufentanil and bupivacaine independently of whether a fixed or variable dose ratio regimen was used, provided that appropriate doses were selected. The ED_{50}s of sufentanil in combination with 80 μg/rat bupivacaine or those of bupivacaine with 0.08 μg/rat sufentanil, as well as the ED_{50}s of both drugs obtained in a fixed dose ratio regimen of 1/1,000 (based on the ED_{50}s of both drugs alone in the TWR test), were always lower than the ED_{50}s of each compound alone and the ED_{50}s theoretically expected on the basis of theoretical additivity. Therefore, under these conditions, a clear synergistic interaction has been demonstrated between epidural sufentanil and bupivacaine. If the concentration ratio of sufentanil/bupivacaine in the fixed dose ratio regimen was changed from 1/1,000 to 1/200 (based on the ED_{50}s of both compounds in the hot-plate test), the ED_{50} of sufentanil for a TWR latency >10 s did not differ from the ED_{50} of sufentanil alone or the theoretically calculated ED_{50} for additivity. For bupivacaine, the difference from bupivacaine alone remains, but here too the difference from the theoretical ED_{50} of additivity disappeared. The data seem to point to a critical constitution of the drug mixtures. Clearly, a minimal amount of nearly 65 μg/rat bupivacaine is needed. These results confirm the findings of our previous study in which no significant change in the ED_{50} of sufentanil could be measured when only 40 μg/rat bupivacaine was added to epidural sufentanil, but the addition of 80 μg/rat bupivacaine resulted in a significant drop in the ED_{50} of sufentanil (23). When the concentration of opioid in the mixture was accentuated, as in the 1/200 ratio compared with the 1/1,000 ratio, the amount of opioid present in a mixture containing sufficient bupivacaine to produce a potentiating effect (which later appeared to be additive) would be so excessive that the potentiation could no longer be demonstrated.

For the hot-plate test, synergism appeared to depend on both the choice of the preset doses in the variable dose ratio method and on the selection of the relative potencies within the fixed dose ratio method. In the variable dose ratio method, the preset doses selected were the same as those used for the TWR testing: 80 μg/rat bupivacaine and 0.08 μg/rat sufentanil. This selected bupivacaine dose appeared to be the maximal inactive dose for the hot-plate test, whereas for sufentanil the selected dose was far below the maximal inactive dose of 0.31 μg/rat. When both selected preset doses were tested, there was a synergistic effect only when bupivacaine was the variable component. The lack of synergism with the preset bupivacaine dose of 80 μg/rat may be explained in terms of a critical amount of compound needed to convert addition into synergism in the hot-plate test. Several results within the present study suggest that this critical dose of bupivacaine is between 99 and 113 μg/rat, being somewhat higher than the critical value in the TWR test.

With regard to the fixed dose ratio method, discrepancies between the results of the TWR and the hot-plate tests were also observed. Although for the hot-plate test the 1/200 dose ratio of sufentanil/bupivacaine was expected to be more appropriate, as suggested by the single ED_{50}s of both compounds, synergism could only be demonstrated with a 1/1,000 ratio, as based upon the relative potency of the two agents in the TWR test. With the 1/200 ratio, there is too much sufentanil present in the drug mixtures relative to the critical dose of bupivacaine necessary to demonstrate synergy.

The results of this study are partly in agreement with previous reports in which synergism was reported for compounds in the TWR test but not in the hot-plate test (15). This may raise questions about the functional relationship between the two behavioral assays used. The TWR test and the hot-plate test clearly differ in response modalities (spinal reflex in the TWR test vs. complex behavioral chain reaction in the hot-plate test). Further, due to its complexity, responding in the hot-plate test is subjected to a larger bias, and reactivity can be masked by partial pain relief (or lateralization) in one hind paw. Therefore, it is our assumption that the hot-plate test may not be the ideal test procedure for demonstrating a spinal interaction. Motor impairment per se cannot account for the differences observed in the two tests, because it has been shown in previous studies that the ED_{50}s for motor paralysis are much higher than those observed here for antinociception in both tests (23).

Although clinical pain states may differ appreciably from the experimental assays studied in the present experiments, the results of the present study may have clinical implications. Synergism makes combinations of extremely low doses of components possible, thus avoiding side effects. However, some critical amount of the compounds might be needed. The evidence of a minimum critical dose for bupivacaine provides arguments that the local anesthetic is potentiated by the opioid if synergism is pursued. A recent meta-analysis (8) demonstrated that bupivacaine–opioid mixtures were better than bupivacaine alone. For the comparison between opioids and opioid–bupivacaine mixtures, less agreement was found. Reasons for this discrepancy may be related in part to the type of surgery concerned but also to the hourly critical dose of bupivacaine. In addition, the fact that there is a minimum critical dose for bupivacaine may indicate that concentrations of bupivacaine in mixtures with opioids should not be reduced too greatly. Several studies have used bupivacaine concentrations as low as 0.01–0.03% (2,4,9). These low concentrations had a dose-sparing effect upon opioid requirements, but there is no simple evidence for synergism.

In summary, a synergistic interaction between epidurally administered bupivacaine and sufentanil may be observed in the TWR test and the hot-plate test in rats when variable and fixed dose ratio methods for drug dosing of the mixtures are used. The synergism could more easily be demonstrated in the former test and may be a purely pharmacodynamic interaction, because in clinical circumstances the addition of bupivacaine does not seem to influence the systemic resorption of the opioid (21). The presence of a minimum critical amount of bupivacaine to obtain synergism may have clinical implications.

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