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Additive interaction between peripheral and central mechanisms involved in the antinociceptive effect of diclofenac in the formalin test in rats

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ABSTRACT

It has been proposed that the antinociception of systemic diclofenac is the outcome of peripheral and central actions. Hence, our purpose was to examine if systemic diclofenac is able to achieve effective concentrations at local and spinal sites and to characterize the interaction between its local and spinal actions. Pain was produced in the rat using the formalin test. Oral diclofenac (1–10 mg/kg) reduced formalin-induced pain. The antinociceptive effect of oral diclofenac (10 mg/kg) was abolished by local or spinal administration of either L-NAME (1–100 μg and 1–50 μg) or glibenclamide (12.5–100 μg and 25–75 μg). These results suggest that oral diclofenac achieves effective concentrations producing an antinociceptive effect involving participation of the NO–potassium channel pathway at both, the local and spinal levels. In an additional experimental series, diclofenac was administered either locally (25–200 μg) or spinally (12.5–100 μg), yielding an antinociceptive effect by both routes. Then, diclofenac was given simultaneously by these two routes in a fixed-ratio, and antinociception was assayed. Isobolographic analysis revealed an additive interaction between the local and spinal effects of diclofenac. Hence, our results provide evidence that the overall antinociceptive effect induced by systemic diclofenac is the outcome of central and peripheral mechanisms.

1. Introduction

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) with potent anti-inflammatory and antinociceptive activity (Todd and Sorkin, 1988). It was initially suggested that its antinociceptive effect was due to inhibition of prostaglandin synthesis at the inflamed tissue (Oliw et al., 1978; Todd and Sorkin, 1988). Notwithstanding, it has been proposed that prostaglandin synthesis inhibition after diclofenac administration is not limited to the periphery, but also occurs at the central level (Vanegas, 2002). Furthermore, the involvement of additional central mechanisms involving spinal and supraspinal actions of endogenous opioids and serotonin, has also been suggested (Björkman, 1995; Sacerdote et al., 1985). At present, the antinociceptive effect of diclofenac after spinal administration has been confirmed in several experimental pain models (Miranda et al., 2001; Jiménez-Andrade et al., 2003).

Tonussi and Ferreira (1994) suggested that the peripheral actions of diclofenac were not limited to prostaglandin synthesis inhibition, but that there was a participation of a direct blockade of inflammatory sensitization involving the participation of nitric oxide (NO). Further observations by our group (Ortiz et al., 2002, 2003), as well as by others (Alves et al., 2004), have established that diclofenac activates the NO–cyclic GMP–potassium channel pathway, yielding peripheral antinociception. On the other hand, we have reported that spinal administration of the selective cyclooxygenase 2 (COX-2) inhibitor lumiracoxib, which is structurally related to diclofenac (Mysler, 2004), produces antinociception by activation of this pathway at both, the peripheral and central levels (Lozano-Cuenca et al., 2005). Therefore, it appears as probable that this is also the case for diclofenac.

It is well documented that diclofenac is rapidly absorbed after administration by the oral, intramuscular and rectal routes, and that it exhibits a low volume of distribution as it is importantly bound to serum proteins (Davies and Anderson, 1997; Todd and Sorkin, 1988). Pharmacokinetic–pharmacodynamic modeling has shown that diclofenac antinociceptive effect after systemic administration is not directly related to plasma concentration, but that the drug must be transferred to its effect compartment in the body (Torres-López et al., 1997). It has been reported that diclofenac is efficiently transferred to the synovial fluid of inflamed joints, with a residence time longer than in plasma (Fowler et al., 1983). Moreover, synovial fluid concentration–effect relationships have been proposed (Davies and Anderson, 1997). Such relationships, however, do not appear representative of what is actually occurring, as they do not consider actions at the

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central level. It has been demonstrated that diclofenac is able to reach the central nervous system after systemic administration, albeit cerebrospinal fluid concentrations are low (less than 10%) concerning those in plasma (Zecca et al., 1991). More recently, Burian et al. (2003) observed in a model of human inflammatory pain that, at comparable injured tissue concentrations, the analgesic effect of systemic diclofenac is significantly greater than that of topical administration. These authors suggested that this is due to the participation of central mechanisms in systemic diclofenac antinociceptive effect. Notwithstanding, the interaction between central and peripheral mechanisms of diclofenac-induced antinociception has not been characterized at present. Hence, the purpose of the present paper was to elucidate if both, central and peripheral mechanisms are activated, and to characterize the interaction between such effects in the overall antinociceptive response of systemic diclofenac in the formalin model of experimental pain in the rat.

2. Materials and methods

2.1. Animals

Female Wistar rats aged 7–8 weeks (weight range, 180–200 g) from our own breeding facilities were used in this study. Animals had free access to drinking water, but food was withheld 12 h prior the initiation of experiments. Efforts were made to minimize animal suffering and to reduce number of animals, which were used only once. At the end of the experiments, rats were sacrificed in a CO_2 chamber. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and the protocol was approved by the Institutional Animal Care and Use Committee. All participating animals were observed regarding behavioral or motor function changes induced by the studied treatments. This behavior was assessed, but not quantified, by testing the animals' ability to stand and walk in a normal posture. All observations were carried out by a blinded investigator.

2.2. Drugs

Diclofenac sodium was a gift of Novartis Farmacéutica (Mexico City). N^G-*L*-nitro-arginine methyl ester (L-NAME), an inhibitor of NO synthase (Rees et al., 1990), was purchased from RBI (Natick, MA, USA). Glibenclamide (glyburide), an ATP-sensitive potassium channel blocker (Edwards and Weston, 1993), was purchased from Sigma (St. Louis, MO, USA). Diclofenac sodium and L-NAME were dissolved in isotonic saline. Glibenclamide was dissolved in dimethyl sulfoxide (20%) and diluted in isotonic saline thereafter. For oral administration, diclofenac was given by means of an intragastric cannula in a volume of 4 ml/kg. For the spinal and local routes, drugs were dissolved in 10 µl and 50 µl of vehicle, respectively. Spinal and local administrations were performed as described previously (Jiménez-Andrade et al., 2003; Lozano-Cuenca et al., 2005).

2.3. Measurement of antinociception

Pain and antinociception were assessed by the formalin test, as previously described (Ortiz et al., 2003). Briefly, 50 µl of diluted formalin (1%) was injected subcutaneously to the dorsal surface of the right hind paw, and the resulting flinching behavior was considered as an expression of nociception. The number of flinches observed during 1-min periods was determined every 5 min, and number of flinches against time curves were constructed. These curves were biphasic. The initial acute phase (0–10 min) was followed by a short quiescent period followed by a prolonged tonic response (15–60 min). The areas under the number of flinches against time curves (AUC) for phase 1 and phase 2 of such curves were calculated by the trapezoidal rule. Then, the percentage of antinociception for each phase was calculated according to the following equation:

$$\text{Percentage of antinociception} = \left[\left(AUC_{vehicle} - AUC_{post\ compound}\right)/AUC_{vehicle}\right] \times 100.$$

Therefore, it is considered that the maximal effect, 100% of antinociception, corresponds to the total suppression of formalin-induced flinches (Jiménez-Andrade et al., 2003; Picazo et al., 2006).

2.4. Determination of peripheral and spinal effects of systemic diclofenac

In order to establish the antinociceptive effect of systemic diclofenac, the drug was given orally at doses ranging from 1 to 10 mg/kg, 30 min before formalin injection. In the second experimental series, oral diclofenac (10 mg/kg) was administered to rats pretreated with a local injection of either L-NAME (1–100 µg) or glibenclamide (12.5–100 µg) in the same site as formalin, 10 min before the insult. Oral diclofenac was considered to elicit peripheral antinociception involving the NO–cGMP–potassium channel pathway if its response was reduced by local L-NAME or glibenclamide (Lozano-Cuenca et al., 2005; Ortiz et al., 2003). In the third experimental series, oral diclofenac (10 mg/kg) was administered to rats pretreated with the spinal administration of either L-NAME (1–50 µg) or glibenclamide (25–75 µg) 10 min before the formalin insult. Oral diclofenac was considered to elicit spinal antinociception involving the NO–cGMP–potassium channel pathway if its response was reduced by intrathecal L-NAME or glibenclamide (Lozano-Cuenca et al., 2005). Drug dosing and times of administration concerning the formalin insult were established based on previous reports (Jiménez-Andrade et al., 2003; Lozano-Cuenca et al., 2005; Ortiz et al., 2003; Picazo et al., 2006).

2.5. Characterization of the interaction between the peripheral and spinal effects of diclofenac

In the first experimental series, diclofenac (at doses ranging from 25 to 200 µg) was injected locally at the same site as formalin, 20 min before the insult. In the second experimental series, diclofenac (at doses ranging from 12.5 to 100 µg) was administered intrathecally (spinally), 10 min before the formalin insult. Percentage of antinociception was plotted against the diclofenac dose. Dose–response curves were constructed by least-squares linear regression, allowing the estimation of ED_{30} values as well as standard error (SE) values according to the goodness of fit of the regression line (Jiménez-Andrade et al., 2003; Picazo et al., 2006).

In an additional experimental series, diclofenac was administrated simultaneously by the local and spinal routes. A dose–response curve was obtained by concurrent diclofenac delivery by the two routes in a fixed-ratio (Tallarida, 2000), based on the ED_{30} values of each individual route. That is, animals received one of the following doses: (local ED_{30} + spinal ED_{30}); (local ED_{30} + spinal ED_{30})/2; (local ED_{30} + spinal ED_{30})/4; (local ED_{30} + spinal ED_{30})/8; and (local ED_{30} + spinal ED_{30})/16. This dose–response curve allowed the determination of the experimental ED_{30} value for the simultaneous local and spinal administrations of diclofenac (Jiménez-Andrade et al., 2003; Picazo et al., 2006).

The interaction between local and spinal diclofenac was characterized by isobolographic analysis (Tallarida, 2000). The theoretical additive ED_{30} was estimated considering that the observed effect with the simultaneous administration by the two routes is the outcome of the sum of the effects of each route. This theoretical ED_{30} was then compared with the experimentally derived ED_{30} value to determine if there was a statistically significant difference (Tallarida, 2002; Tallarida et al., 1999). The theoretical and experimental ED_{30} values

for the simultaneous local and spinal diclofenac administrations were also contrasted by calculating the interaction index (γ) as follows:

$\gamma = ED_{30}$ experimental/ED_{30} theoretical.

An interaction index not significantly different from unity corresponds to an additive interaction, whereas values higher and lower than unity imply an antagonistic and synergistic interaction, respectively (Tallarida, 2002).

2.6. Statistical analysis

Data are presented as mean±SEM for 6 animals per group. Comparisons between treatments were performed by one-way analysis of variance (ANOVA) with Tukey's test for *post-hoc* comparison. Statistical significance between the theoretical additive point and the experimentally derived ED_{30} value was evaluated using the Student's *t* test, as described by Tallarida (2000, 2002). Differences are considered to be significant when $p<0.05$.

3. Results

3.1. Peripheral and spinal effects of oral diclofenac

Subcutaneous injection of formalin into the hind paw produced a typical pattern of flinching behavior. The first phase of flinching started immediately after administration of formalin and then vanished gradually in 10 min. The second phase started at 15 min and lasted for at least 1 h (Jiménez-Andrade et al., 2003; Ortiz et al., 2003; Picazo et al., 2006). Oral administration of diclofenac (1–10 mg/kg) produced a dose-dependent reduction in the flinching behavior otherwise observed after formalin injection. Diclofenac significantly reduced the number of flinches during phase two, but not during phase one (Fig. 1). When oral diclofenac was given to rats pretreated with the local injection of either L-NAME or glibenclamide, the antinociceptive effect was abolished in a dose-dependent manner (Fig. 2). Similarly, when oral diclofenac was given to rats pretreated by the spinal administration of either L-NAME or glibenclamide, the antinociceptive effect of diclofenac was abolished in a dose-dependent manner (Fig. 3). Local or spinal administration of either L-NAME or glibenclamide, without oral diclofenac, did not produce any significant change in formalin-induced flinching behavior (Figs. 2 and 3).

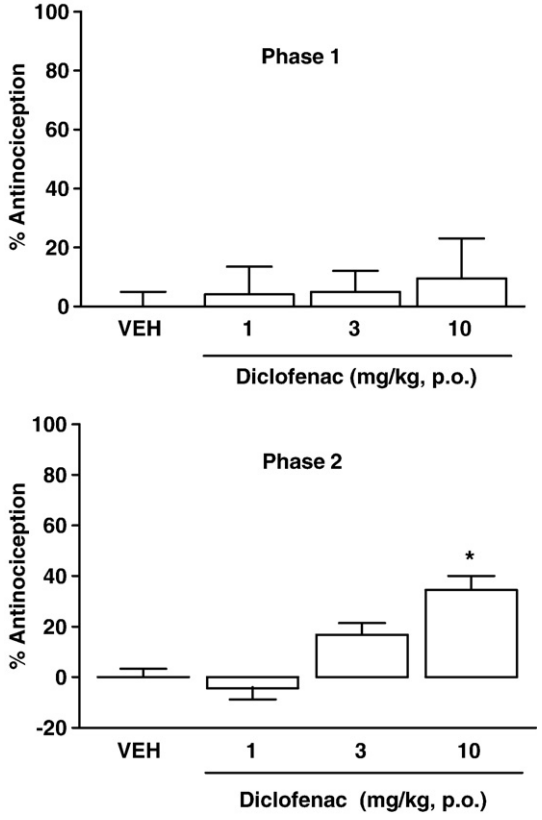

Fig. 1. Antinociceptive effect of oral (p.o.) administration of diclofenac in phase 1 (upper panel) and phase 2 (lower panel) of the formalin test. Data are expressed as mean±SEM of 6 animals. *Significantly different from vehicle (VEH). Statistical analysis was performed by analysis of variance followed by Tukey's test. Differences were considered to reach statistical significance when $p<0.05$.

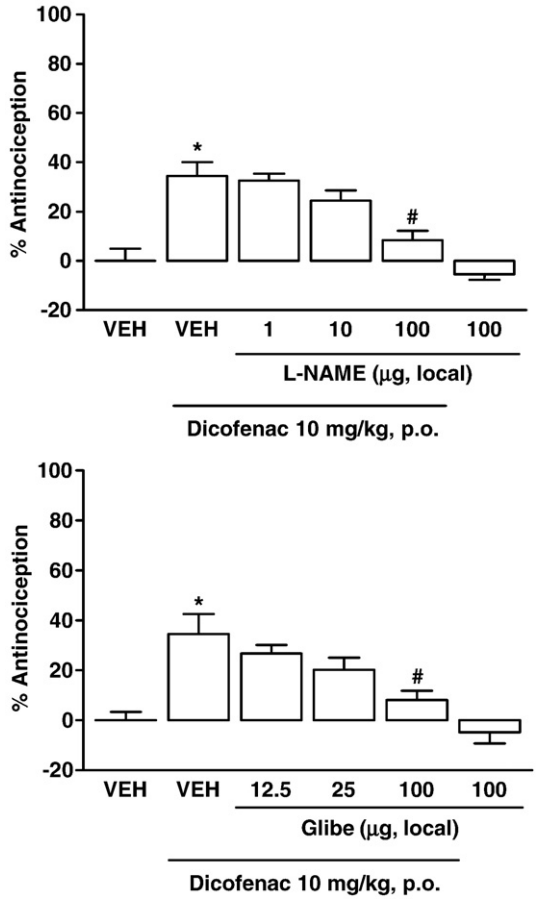

Fig. 2. Effect of local pretreatment with either L-NAME (upper panel) or glibenclamide (Glibe, lower panel) on the antinociceptive effect of oral (p.o.) diclofenac in the second phase of the formalin test. Data are expressed as mean±SEM of 6 animals. *Significantly different from vehicle (VEH). #Significantly different from diclofenac plus vehicle. Statistical analysis was performed by analysis of variance followed by Tukey's test. Differences were considered to reach statistical significance when $p<0.05$.

3.2. Interaction between the local and spinal effects of diclofenac

Local and spinal administrations of diclofenac failed to induce any significant reduction in flinching behavior during the first phase of the formalin test (data not shown), but produced a dose-dependent antinociceptive effect in the second phase of the assay (Fig. 4, upper and middle panels). This allowed the estimation of the ED_{30} values for each of these routes; being 72.2±12.5 µg and 63.7±5.0 µg for local and spinal diclofenac, respectively. Fixed-dose ratio combinations of local and spinal diclofenac were prepared based on ED_{30} values, as described above, and assayed in order to construct the dose–response curve for the simultaneous administration of diclofenac by these two routes (Fig. 4, lower panel). The actually observed (experimental) ED_{30} value was 56.8±8.8 µg.

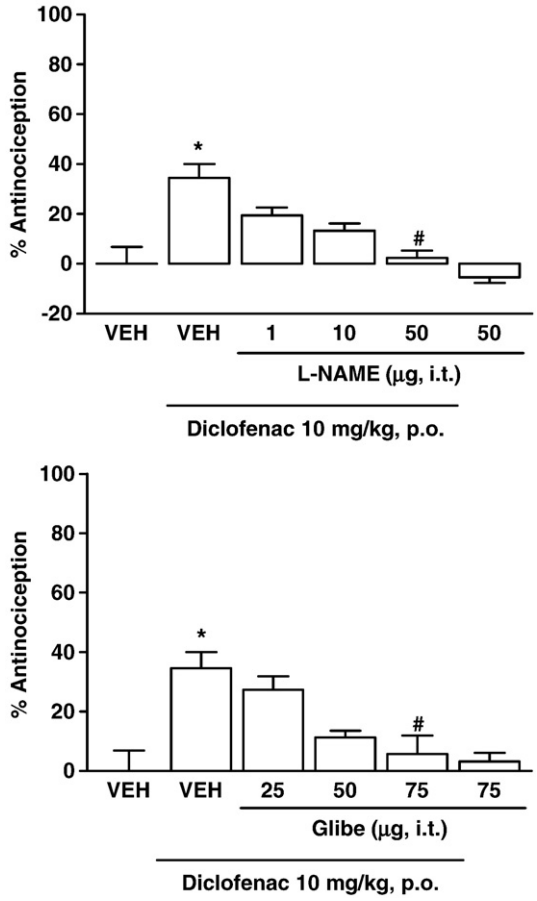

Fig. 3. Effect of spinal (intrathecal, i.t.) pretreatment with either L-NAME (upper panel) or glibenclamide (Glibe, lower panel) on the antinociceptive effect of oral (p.o.) diclofenac in the second phase of the formalin test. Data are expressed as mean ± SEM of 6 animals. *Significantly different from vehicle (VEH). #Significantly different from diclofenac plus vehicle. Statistical analysis was performed by analysis of variance followed by Tukey's test. Differences were considered to reach statistical significance when $p<0.05$.

Fig. 5 shows the isobologram for the interaction of the local and spinal effects of diclofenac. The theoretical ED_{30} value can be appreciated on the line corresponding to a purely additive interaction (Tallarida, 2000), being 67.9 ± 6.7 µg. As it can be appreciated in Fig. 5, the experimental ED_{30} value was located below, but close to the additive interaction line. When the theoretical and experimental ED_{30} values were compared by the Student's *t* test ((Tallarida, 2000, 2002), no statistically significant difference was detected ($p=0.97$). Furthermore, the estimated interaction index (γ) was 0.8 ± 0.2, being not statistically significantly different from unity ($p=0.08$).

4. Discussion

It was initially suggested that diclofenac elicits antinociception by the sole activation of peripheral mechanisms of action (Oliw et al., 1978; Todd and Sorkin, 1988; Tonussi and Ferreira, 1994). Hence, it was supposed that, after systemic administration, diclofenac only required to be transferred to the inflamed tissue (Davies and Anderson, 1997; Fowler et al., 1983). Moreover, concentration–effect relationships were established for diclofenac in the injured tissue site where sampling was possible, such as the synovial fluid of inflamed joints. Notwithstanding, central actions of diclofenac have also been proposed (Björkman, 1995; Burian et al., 2003; Jiménez-Andrade et al., 2003; Sacerdote et al., 1985; Vanegas, 2002). The participation of central mechanisms, however, was not clear since, although it has been demonstrated that diclofenac is able to cross the blood-brain barrier, concentration in cerebrospinal fluid observed after systemic diclofenac is considerably lower than those present in plasma (Zecca et al., 1991). This suggests that the role of central mechanisms in the overall effect of systemic diclofenac could be limited.

We have previously reported that the injection of diclofenac at the injury site results in antinociception in the formalin experimental pain model. This response is likely due to a purely local (peripheral) action, as diclofenac administration in the contralateral paw, with respect to formalin, fails to produce antinociception. This allows discarding a systemic drug effect (Jiménez-Andrade et al., 2003; Ortiz et al., 2003). Furthermore, it has been observed that the peripheral antinociceptive effect of diclofenac can be abolished by local administration of inhibitors of the NO–cGMP–potassium channel pathway (Ortiz et al., 2003). In this study, we observed that the antinociceptive effect of oral diclofenac was abolished in a dose-dependent manner by the local injection of either L-NAME, an inhibitor of NO synthase, or by the local injection of glibenclamide, an ATP-sensitive potassium channel blocker. These observations suggest that diclofenac distributes to the

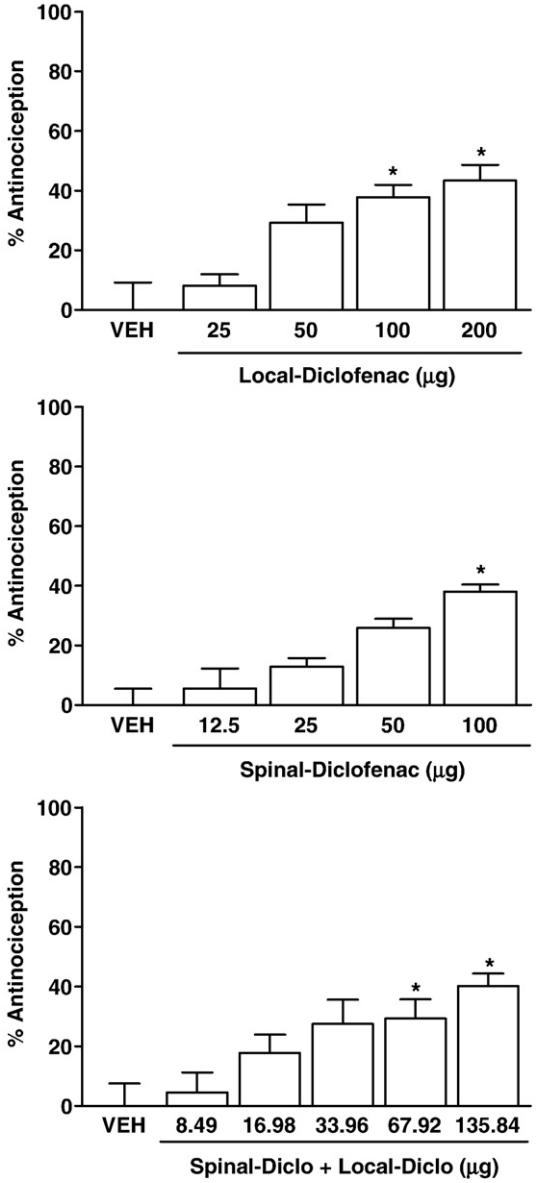

Fig. 4. Antinociceptive effect of local (upper panel), spinal (middle panel) and spinal + local (lower panel) administrations of diclofenac (Diclo). Data are expressed as mean ± SEM of 6 animals. *Significantly different from vehicle (VEH). Statistical analysis was performed by analysis of variance followed by Tukey's test. Differences were considered to reach statistical significance when $p<0.05$.

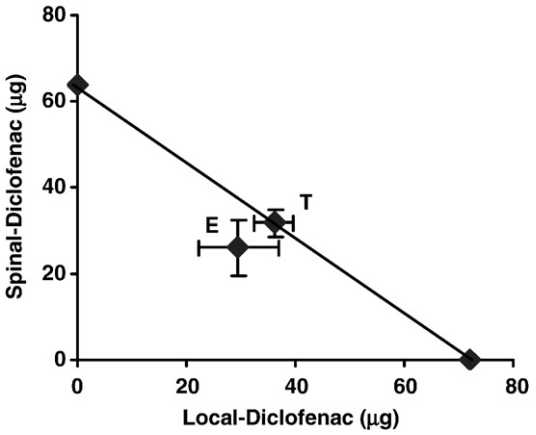

Fig. 5. Isobologram showing the interaction between the antinociceptive effects of local and spinal diclofenac in the formalin test. The points located in the *x* and *y* axes depict the ED_{30} values for the local and spinal routes respectively. The oblique line corresponds to the theoretical additive line. The point located in the middle of this line "T" is the theoretical additive ED_{30} value estimated from the individual effects of each route assuming a purely additive interaction. The point indicated by "E" corresponds to the experimentally determined ED_{30} value after the simultaneous administration of diclofenac by the local and spinal routes. Horizontal and vertical bars indicate SEM. There was no statistically significant difference between "T" and "E" ($p>0.05$), as determined by the Student's *t* test. See text for details.

injured tissue site achieving sufficiently high concentrations to induce a peripheral antinociceptive response involving activation of the NO–cGMP–potassium channel pathway. This interpretation is consistent with reports describing an efficient diclofenac transfer from the circulation to the inflamed tissue (Davies and Anderson, 1997; Fowler et al., 1983). However, we also observed that the antinociceptive effect of oral diclofenac could be abolished by the spinal administration of either L-NAME or glibenclamide. These results suggest that, after systemic administration of this NSAID, a certain drug amount is able to be transferred through the blood-brain barrier, reaching spinal sites of action and eliciting an antinociceptive response involving activation of the NO–cGMP–potassium channel pathway at the central level. Our results, therefore, are consistent with the assumption of Burian et al. (2003) on a participation of central mechanisms in the antinociceptive effect of systemic diclofenac in addition to peripheral actions.

Diclofenac was able to elicit an antinociceptive effect in the formalin test after either local or spinal administration. Hence, it appears that diclofenac, like lumiracoxib, a molecule to which it is structurally related (Mysler, 2004), is able to elicit both, peripheral and spinal antinociceptions (Lozano-Cuenca et al., 2005). Moreover, simultaneous administration of diclofenac by these two routes also resulted in dose-dependent antinociception. Isobolographic analysis (Tallarida, 2000, 2002) showed that the interaction between the spinal and local effects of diclofenac is additive. That is, the effect of diclofenac given by both routes is the result of the sum of spinal and local effects. Taken together, our observations can be interpreted as follows. After oral administration (or by any systemic route), diclofenac is absorbed, reaching the circulation, and then shows a reduced distribution (Todd and Sorkin, 1988). The volume of distribution, albeit being low, includes the effect compartment of the drug (Torres-López et al., 1997). The effect compartment includes the inflamed tissue site (Davies and Anderson, 1997; Fowler et al., 1983). Nonetheless, diclofenac is also transferred to the central nervous system across the blood-brain barrier. Hence, the effect compartment does not only include the inflamed tissue in the periphery, but also central sites of action (Burian et al., 2003; Jiménez-Andrade et al., 2003; Vanegas, 2002).

It should be noted that diclofenac transfer across the blood-brain barrier is limited, concentrations achieved in the cerebrospinal fluid being about 10% of those in plasma (Zecca et al., 1991). Hence, the concentrations achieved after diclofenac systemic administration appear to be too low concerning the reported doses, which elicit antinociception when the drug is directly administered into the central nervous system (Vanegas, 2002). In fact, in the present work, the doses required for spinal antinociception were similar to those which produce the local response. As diclofenac is transferred more efficiently to the inflamed tissue, where in fact it accumulates (Fowler et al., 1983), than to the central nervous system (Zecca et al., 1991), our results could be against a significant participation of central mechanisms in the antinociceptive effect of systemic diclofenac. Nonetheless, our observation that there is an additive interaction between the central and peripheral actions of diclofenac conciliates the fact of a limited diclofenac transfer to central sites with a significant participation of central mechanisms. Diclofenac concentrations at central sites of action need not to be high, provided that the NSAID is also present at the peripheral sites of action, since the overall antinociceptive response results from the sum of central and peripheral mechanisms.

In the present work we used the formalin test in the rat. It has been reported that oral, local and spinal diclofenac yield a dose-dependent antinociceptive effect in this assay, although with a limited efficacy; the maximal effect being less than 50% of antinociception (Jiménez-Andrade et al., 2003). Notwithstanding, the formalin experimental pain model appeared to be suitable for our purposes. Activation of the NO–cGMP–potassium channel pathway by several drugs at either the spinal or peripheral levels results in antinociception (Lozano-Cuenca et al., 2005; Mixcoatl-Zecuatl et al., 2006; Ortiz et al., 2003). Hence, inhibitors of NO synthase or of soluble guanylyl cyclase, as well as potassium channel blockers can be used to abolish systemic diclofenac-induced antinociception and, hence, to identify the sites of action of this drug without measuring diclofenac plasma concentrations at the local and spinal levels, which will certainly interfere with the results of nociception in the formalin test (Capone and Aloisi, 2004). It should be mentioned, however, that our results cannot be directly extrapolated to the clinical situation. Our experimental design did not allow examining the role of central and peripheral prostaglandin synthesis inhibition in diclofenac-induced antinociception. Furthermore, we did not explore supraspinal mechanisms of action of diclofenac, which have also been proposed to play a role in antinociception (Björkman, 1995; Sacerdote et al., 1985). Notwithstanding, the present observations are of relevance as they allow a better understanding of diclofenac effects *in vivo*, providing a rational basis for the role of central mechanisms of antinociception, despite a limited diclofenac transfer across the blood-brain barrier.

It has been demonstrated that for evaluation of the interaction between analgesic drugs isobolographic analysis is a convenient tool (Tallarida, 2000). Thus, from the dose–response curves of each individual agent, the dose resulting in 50% of the effect (ED_{50}) can be determined and used to realize the drugs combinations. However, considering a maximal effect of 100% as the total suppression of formalin-induced flinches, it appeared that at the doses used in the present study diclofenac was unable to achieve a 50% response, and thus the calculation of ED_{50} was not feasible. Therefore, we estimated the ED_{30} instead of ED_{50} for both routes. The election of an ED minor or different from ED_{50} has shown to be a convenient tool for isobolographic analysis (Ossipov et al., 1990; Tallarida, 1992; Tallarida et al., 1999; Jiménez-Andrade et al., 2003; Granados-Soto and Argüelles, 2005; Picazo et al., 2006; Bhat et al., 2007). Indeed, we have used higher diclofenac doses in previous studies and obtained different ED_{30} (Jiménez-Andrade et al., 2003; Picazo et al., 2006). This was due to the fact that in those studies we studied diclofenac-induced analgesia in the 5% formalin test, while in the present study we used 1% formalin. We have shown that 1% formalin produces a lower pain level than 5% formalin and hence antinociception can be achieved with lower diclofenac doses (Torres-López et al., 2002). This is why there are differences in ED_{30} values between the present study and our previous works. It should be considered that our aim was to characterize the interaction between the peripheral and spinal effects of diclofenac by isobolographic analysis. With the used dose range we

were able to calculate ED_{30} values for the local and spinal routes, and for the combination of both routes. These data allowed to perform the isobolographic analysis and to conclude that, under these conditions, the interaction between the peripheral and spinal actions of diclofenac is additive. Of course, we are convinced that the reported data are only valid for the 1% formalin model and cannot be extrapolated to other models of nociception using different experimental conditions.

In summary, the present results provide evidence for an additive interaction between the central and peripheral mechanisms in the overall antinociceptive response of diclofenac after systemic administration. An integrative approach, considering all mechanisms playing a role in the antinociception, and of the pharmacokinetic factors involved, will certainly lead to a better perspective of diclofenac clinical efficacy and safety.

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