

B Vitamins Increase the Analgesic Effect of Ketorolac in the Formalin Test in the Rat

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ABSTRACT

The purpose of this study was to assess the possible synergistic interaction between ketorolac and B-vitamins in the rat formalin test. Female Wistar rats were injected into the dorsal surface of the right hind paw with 50 μ l of diluted formalin (1%). Reduction of the number of flinches was considered as antinociception. Ketorolac (0.32-10 mg/kg, po), B-vitamins (56-316 mg/kg), or a combination of B-vitamins (either 100:100:1 or 100:100:5 proportion of vitamin B₁, B₆ and B₁₂, respectively) and ketorolac was administered orally and the antinociceptive effect was determined. Isobolographic analyses were used to define the nature of the functional interactions between B-vitamins and ketorolac in a proportion (0.5:0.5, ketorolac:B-vitamins). Ketorolac (ED₂₅ 3.5 $\pm$ 0.7 mg/kg), B-vitamins in a 100:100:1 (ED₂₅ 277.1 $\pm$ 30.2 mg/kg) or 100:100:5 (ED₂₅ 157.3 $\pm$ 13.7 mg/kg) proportion and fixed-ratio B-vitamins-ketorolac combinations dose-dependently reduced flinching behavior during second phase of the test. Theoretical ED₂₅ values for the combination ketorolac-B vitamins estimated from the isobolograms were 140.3 $\pm$ 15.1 and 80.4 $\pm$ 6.8 mg/kg for 100:100:1 and 100:100:5 proportions, respectively. These values were significantly higher than experimental ED₂₅ values which were 73.3 $\pm$ 6.3 and 47.7 $\pm$ 6.4 mg/kg for 100:100:1 and 100:100:5 proportions, respectively. Results indicate that oral ketorolac and B-vitamins can interact synergistically to reduce inflammatory pain in the formalin test and suggest the use of those combinations to relief this kind of pain in humans.

INTRODUCTION

B vitamins have been used as analgesic drugs to treat pain disorders associated with their deficiency [1]. In addition, B vitamins have been reported to be clinically useful alone or combined with non-steroidal anti-inflammatory drugs (NSAIDs) in some painful disorders such as polyneuropathy [2], neuritis [3], painful vertebral syndrome [4] or rheumatic diseases [5]. There is evidence that B vitamins are able to increase the antinociceptive activity of diclofenac in animals [6-7]. In humans, B vitamin preparations

have been extensively used as analgesic adjuvant combined with diclofenac for the treatment of acute lumbar vertebral pain [4,8]. However, since some negative results have also been reported [9-11] the adjuvant properties of the B vitamins complex are not completely established. In the present study, we evaluated the analgesic activity of ketorolac alone and combined with the B vitamins complex (B₁, B₆ and B₁₂) using the formalin test in the rat.

METHODS: Female Wistar rats of 7-8 weeks (weight range, 180-200 g) from our own breeding facilities were used in this study. Rats had free access to drinking water, but food was restricted 12 hr before the experiment. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals [12]. Additionally, the study was approved by the Institutional Animal Care and Use Committee (Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, México, DF, MEXICO).

Evaluation of antinociceptive activity: Antinociception was assessed by the formalin test. Rats were placed in an open Plexiglas® observation chamber for 30 min to allow them to accommodate to their surroundings, then they were removed for formalin administration. The rats were injected into the dorsal surface of the right hind paw with 50 μ l of dilute formalin (1%), using a 30-gauge needle. The animal was then returned to the chamber for observation. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. The rats were observed for nociceptive behavior immediately after formalin injection.

Nociceptive behavior was quantified as the number of flinches of the injected paw during 1 min periods every 5 min up to 60 min after injection. Flinching was readily discriminated and was characterized as rapid and brief withdrawal or flexing of the injected paw. As reported, formalin-induced flinching behavior was biphasic [13]. The initial acute phase (0-10 min) is followed by a relatively short quiescent period, which is then followed by a prolonged tonic response (15-60 min). At the end of the experiment, rats were sacrificed in a CO₂ chamber.

Drugs: Ketorolac was purchased from Sigma (St. Louis, MO, USA). B vitamins were kindly provided by Merck S.A. de C.V. (Mexico City). All drugs were dissolved in saline.

Study design: Association of thiamin, pyridoxine and cyanocobalamin was used in a proportion of 100:100:1 and

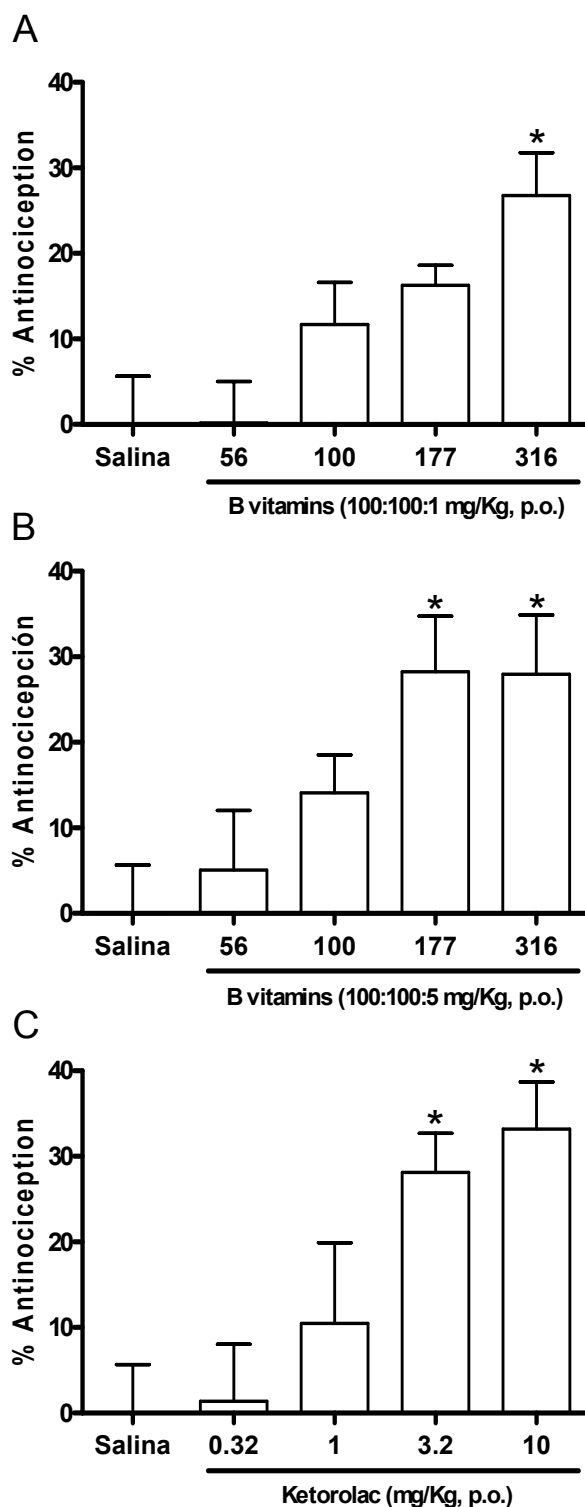


Figure 1. Antinociceptive effect observed after oral administration of ketorolac (A) and the mixture of B vitamins either 100:100:1 proportion (B) or 100:100:5 proportion (C) on the 1% formalin test. Rats were orally pretreated with vehicle (VEH) or drugs before formalin injection. Data are expressed as % of antinociception. Bars are the means $\pm$ SEM for 6 animals. * Significantly different from the vehicle group ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

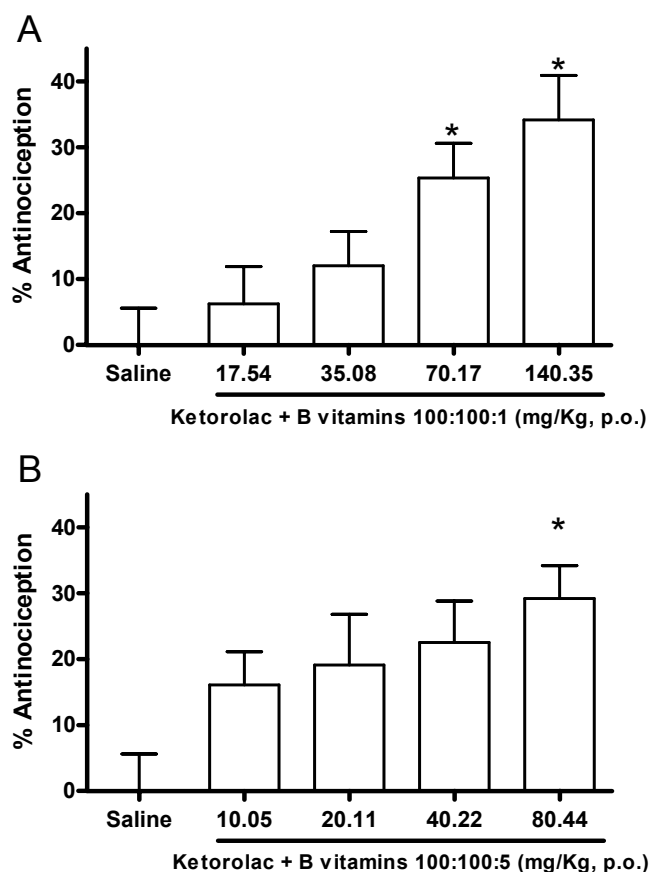


Figure 2. Antinociceptive effect observed after oral administration of the combination of ketorolac and B vitamins either at a 100:100:1 (A) or 100:100:5 (B) proportion on the 1% formalin test. Rats were orally pretreated with vehicle (VEH) or the combinations before formalin injection. Data are expressed as % of antinociception. Bars are the means $\pm$ SEM for 6 animals. * Significantly different from the vehicle group ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

100:100:5. This means that a dose of 100 mg/kg of thiamin-pyridoxine-cyanocobalamin indicates 100 mg/kg of thiamin, 100 mg/kg of pyridoxine and 1 mg/kg of cyanocobalamin or 5 mg of cyanocobalamin, respectively. Rats received the oral administration of saline (4 ml/kg) or increasing doses of B vitamins (56-316 mg/kg, either a 100:100:1 or 100:100:5 proportion of vitamin B₁, B₆ and B₁₂, respectively), ketorolac (0.32-10 mg/kg) or dose-fixed ratio combinations of B vitamins and ketorolac 10 min before formalin injection. After formalin injection, flinching behavior was assessed for the next 60 min.

Data analysis and statistics: All results are presented as means $\pm$ SEM for at least 6 animals per group. The area under the number of flinches against time curves (AUC) was calculated by the trapezoidal rule [14]. Dose-response curves for each compound tested were established based on the percent maximum possible effect (expressed as % Antinociception) calculated from AUC of the phase 2 of each individual rat: % Antinociception = ((vehicle-post-compound/vehicle) $\times$ 100). For evaluation of the interaction between ketorolac and B vitamins, isobolograms were

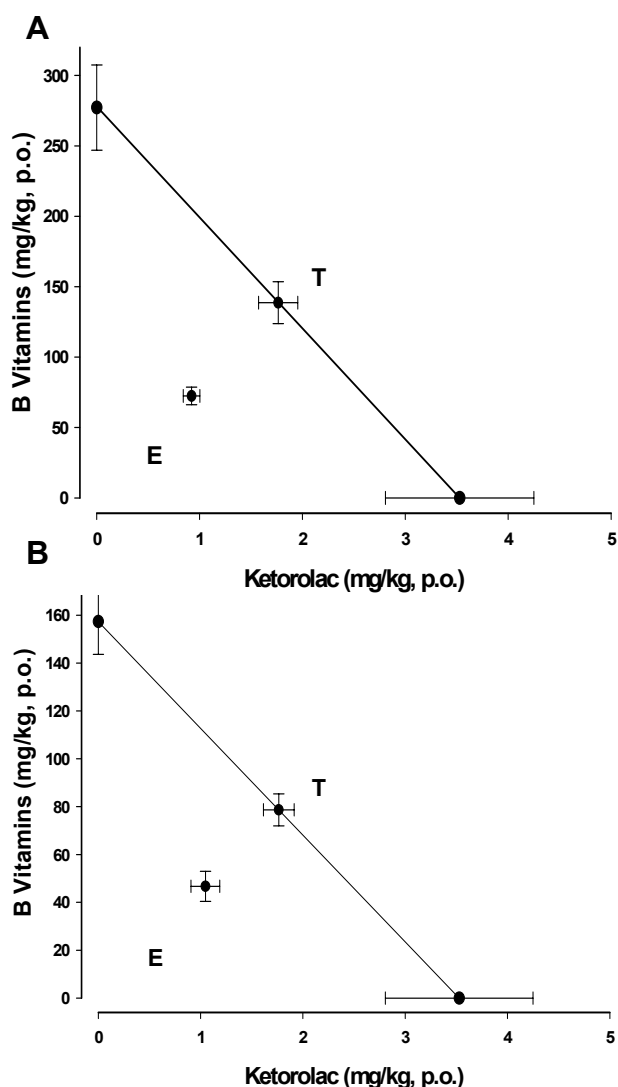


Figure 3. Isobolograms showing the synergistic interaction between ketorolac and B vitamins either at a 100:100:1 (A) or 100:100:5 (B) proportion on the 1% formalin test. Horizontal and vertical bars indicate SEM. The oblique line between the x axis and y axis is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the separate ED_{25} values. In both cases, the experimental point lies far below the additive line, indicating a significant synergism ($P < 0.05$).

constructed using ED_{25} values obtained when the drugs were administered alone or combined. We used ED_{25} instead of ED_{50} values because neither drug was able to reach more than 33% of antinociception in our model [14]. The construction of the dose-response curves and the determination of doses producing 25% maximum possible effect (ED_{25}) were computed. To perform the isobolographic analysis, ketorolac and B vitamins were administered in combination as fixed ratios of equi-effective ED_{25} dose for each drug (ketorolac/B vitamins=0.5:0.5). The ED_{25} values ($\pm$ SEM) for ketorolac and B vitamins alone were plotted on the x and y axes, respectively, and the theoretical additive point was calculated according to Tallarida et al [14]. From the dose-

response curve of the combined drugs, the ED_{25} value of the total dose of the combination was calculated. Statistical significance between the theoretical additive point and the experimentally derived ED_{25} value was evaluated using Student's *t* test. An experimental ED_{25} significantly less than the theoretical additive ED_{25} ($P < 0.05$) was considered to indicate a synergistic interaction between ketorolac and B vitamins.

RESULTS: Formalin administration produced a typical pattern of flinching behavior. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at 15 min and lasted until 1 hr. Oral administration of B vitamins (56-316 mg/kg) dose-dependently reduced flinching behavior induced by formalin during the phase 2 (Figure 1) reaching a maximum effect of 26 (ED_{25} 277.1 $\pm$ 30.2 mg/kg) and 28% (ED_{25} 157.3 $\pm$ 13.7 mg/kg) for the 100:100:1 and 100:100:5 proportion, respectively. In addition, oral administration of ketorolac (0.32-10 mg/kg) significantly reduced formalin-induced nociceptive behavior in the rat, reaching a maximum effect of 33% (ED_{25} 3.5 $\pm$ 0.7 mg/kg).

Oral coadministration of ketorolac and B vitamins induced a dose-dependent increase in the % antinociception (Fig. 2). The antinociceptive effect of the combination was observed mainly on the second phase of the test. Theoretical ED_{25} values for the combination ketorolac-B vitamins estimated from the isobolograms were 140.3 $\pm$ 15.1 and 80.4 $\pm$ 6.8 mg/kg for 100:100:1 and 100:100:5 proportions, respectively. These values were significantly higher than experimental ED_{25} values which were 73.3 $\pm$ 6.3 and 47.7 $\pm$ 6.4 mg/kg for 100:100:1 and 100:100:5 proportions, respectively. As shown in figure 3, the experimentally derived ED_{25} ($\pm$ SEM) in the isobologram are below the theoretically additive dose line, indicating a significant synergistic interaction between ketorolac and B vitamins in the formalin test ($P < 0.05$).

DISCUSSION: The present study demonstrated that oral administration of ketorolac and the mixture of B vitamins (100:100:1 or 100:100:5 proportion of B₁, B₆ and B₁₂, respectively) is able to significantly reduce formalin-induced nociception in the rat. We also demonstrated in this work that orally co-administered ketorolac and B vitamins have a synergistic antinociceptive interaction against noxious stimuli produced by 1% formalin. Ketorolac produced a significant antinociceptive effect as previously demonstrated in inflammatory pain models [15-19] and human beings [20-21]. In addition, oral

administration of the mixture of B vitamins also produced antinociception in the formalin test. However, the antinociceptive effect observed with B vitamins was less than 40% of the maxim possible effect in the test. These results agree with our previous observation that B vitamins are able to significantly reduce the pain induced by formalin in the rat [22-25] and confirm that B vitamins are effective to diminish inflammatory pain. In our work, we observed that the 100:100:5 proportion was slightly better than the 100:100:1 proportion to reduce inflammatory pain in the rat as greater doses of the 100:100:1 proportion were needed to reach the same reduction in pain. When co-administered, lower doses of ketorolac and B vitamins were necessary to reach an antinociceptive effect of 25%. In addition, isobolographic analyses showed a synergistic interaction between ketorolac and the mixture of B vitamins either in 100:100:1 or 100:100:5 proportions. Previously we have found a synergistic interaction between the mixture of B vitamins and gabapentin [26], diclofenac [24-25], acetaminophen and metamizol, but not nimesulide.

The mechanism of the antinociceptive interaction between these drugs remains to be elucidated. The mechanism of the synergy observed could be due to the different sites of action of ketorolac and B vitamins. In the case of ketorolac, there is evidence that this drug produces its antinociceptive effect through the inhibition of cyclooxygenase 1 [27]. However, other observations suggest that ketorolac could also activate the nitric oxide-cyclic GMP-K⁺ channel pathway in order to reduce pain in the rat [17,28]. On the other hand, B vitamins are able to diminish pain through the activation of afferent inhibitory control of nociceptive neurons at the spinal cord [29]. In addition, our group has found that the antinociception induced by B vitamins can be partially reduced by naloxone and L-NAME, but not by D-NAME or methiothepin thus suggesting that opioid and nitrenergic, but not serotonergic, mechanisms could participate in their antinociceptive effect [24-25].

In summary, we have found that ketorolac and the mixture of different proportions of B vitamins are able to reduce inflammatory pain in the formalin test. In addition, a synergistic interaction was observed between ketorolac and B vitamins, thus suggesting that this combination could be useful to treat inflammatory pain in humans.

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