# Evaluation of the formalin test to assess the analgesic activity of diflunisal in the rat

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## Abstract

The formalin test was evaluated to assess the analgesic activity of diflunisal in the rat. Fifty microliters of a 5% formalin solution was injected into the hindpaw of rats and two distinct nociceptive behaviors, i.e. flinching/shaking and licking/biting of the injected paw, were recorded over 120 min. The effect of factors such as age of the animal, time of injection (morning vs. afternoon), site of injection (right vs. left hind paw) were evaluated. Both nociceptive behaviors exhibited a biphasic time course. Rats weighing 210–220 grams showed a more intense response compared to older rats weighing 240–250 or 270–280 grams. The nociceptive behavior response was affected by the time of formalin injection and was more pronounced in the morning. Diflunisal (100 mg/kg, i.v. infusion over 3 min) caused a significant delay in the flinching/shaking response vs. time curve, whereas the licking/biting response was significantly inhibited. When carried out under carefully controlled conditions, the formalin test may be useful to study the analgesic effect of diflunisal in the rat. It seems to be less sensitive, however, than other commonly used nociceptive tests. © 1998 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Subcutaneous injection of a dilute aqueous formalin (formaldehyde) solution into the dorsal surface of the rat or mouse hind paw elicits two distinct quantifiable nociceptive behaviors, i.e. flinching/shaking and licking/biting of the injected paw (Dubuisson and Dennis, 1977; Tjolsen et al., 1992). This formalin-induced nociceptive behavior shows an early and a late phase. The early phase, which starts immediately following injection of formalin, only lasts approximately 5 min and is probably due to direct chemical stimulation of nociceptors (acute pain). The second phase, which lasts 20 to 40 min, starts approximately 15 to 20 min following formalin injection and experimental data suggest that peripheral, inflammatory processes are involved (Haley et al., 1989). The formalin test differs from most other nociceptive tests, such as the hot plate, tail flick and tail pinch tests, in that it enables evaluation of analgesic activity towards moderate, continuous pain generated by injured tissue. As a result, it has been suggested that this test provides a more valid model for clinical pain compared to the threshold or reflex tests such as the hot plate and tail flinch tests (Dubuisson and Dennis, 1977; Abbott et al., 1981; Alreja et al., 1984).

The aim of the present study is to evaluate the formalin test to assess the analgesic activity of drugs in the rat and its potential usefulness in pharmacokinetic/pharmacodynamic studies with NSAID analgesics in the rat. More specifically, the reproducibility of the formalin test was studied and preliminary experiments were carried out using diflunisal, a salicylate derivative with antipyretic, analgesic and anti-inflammatory activities (Stone et al., 1977).

## 2. Materials and methods

### 2.1. Chemicals

Diflunisal, naproxen and formalin (10% solution) were purchased from Sigma Chemical Co. (St. Louis, MO).

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Tetramethylammonium sulphate was obtained from Aldrich Chemical Co. (Milwaukee, WI) and pentobarbital (Nembutal[R] 60 mg/ml for veterinary use) from Sanofi (Brussels, Belgium). Acetonitrile and methanol were HPLC grade (Labscan, Dublin, Ireland). All other reagents were of analytical grade.

## 2.2. Experimental animals

A series of preliminary experiments was carried out on male Wistar rats of variable weight (210–220 g, 240–250 g, 270–280 g) obtained from the university breeding facilities. Animals were housed two to a single cage in an animal room maintained at a constant temperature (20–22°C) with a 12 h alternating light/dark cycle. The animals had free access to a normal diet for laboratory rats (U.A.R., Epinay-sur-Orge, France) and water. Since the formalin test was carried out in a dedicated room with no other human activity, the animals were accustomed during the daytime to this new environment for at least 2 days prior to the experiment. The temperature in this room was also maintained between 20 and 22°C. The day of the experiment the rats were housed individually in the observation chamber (L 26 cm; W 21 cm; H 15 cm) for at least 1 h before testing. During this period as well as during the actual test, the animals did not have access to food or water. All experimental procedures in rats were approved by the University Animal Experimentation Ethics Committee and adhered to the Principles of Laboratory Animal Care.

## 2.3. Formalin test

After the acclimation period, 50 µl of a 5% formalin solution was injected subcutaneously into the dorsal surface of the right or left hind paw of the rat using a 29 gauge needle (U-100 Insulin Syringe, Beckton and Dickinson, Dublin, Ireland). The 5% formalin solution was prepared by dilution with normal saline of the commercial 10% formalin solution. The total number of flinches of the hind paw and/or the hind quarters was recorded by visual observation for 5 min periods for a total observation duration of 2 h following injection of formalin. In addition, licking/biting of the injected paw was recorded using a digital time-out stopwatch as total licking time (s) per 5 min observation period for a total duration of 2 h following injection of formalin. Control rats, injected subcutaneously in the dorsal surface of the right hindpaw with 50 µl normal saline, did not show these typical nociceptive behaviors. The influence of the following experimental conditions was evaluated: body weight of the animal (210–220 g, 240–250 g, 270–280 g), time of day of formalin injection (10:00 a.m. versus 2:00 p.m.) and formalin injection in the right versus the left hind paw. In these studies groups of 8 rats were used.

## 2.4. Evaluation of the analgesic effect of diflunisal using the formalin test

The day before the formalin test, cannulae were implanted under pentobarbital anesthesia (50 mg/kg i.p.) in the right and left jugular veins of male Wistar rats weighing 210–220 grams (Brunelle and Verbeeck, 1997). The next day, diflunisal (20 or 100 mg/kg) was administered by short i.v. infusion (3 min) of approximately 2 ml of the diflunisal solution (2 or 10 mg/ml in phosphate buffer pH 7.4). To control rats 2 ml of vehicle (phosphate buffer ph 7.4) were administered by short i.v. infusion or by i.p. injection. All diflunisal administrations took place at 9:30 a.m., 30 min prior to formalin injection into the right hind paw. Groups of 6 rats were used in this series of experiments.

## 2.5. HPLC assay of diflunisal in rat plasma

Blood samples (200 µl) were withdrawn via the second jugular cannula just before and at the following times after (the start of) diflunisal administration: 1.5, 3, 10, 15, 25, 40, 60, 90, 120, 150, 180, 240, 300, 360, 420 and 480 min. Each 200 µl blood sample was replaced by an equal volume of isotonic saline solution. Blood samples were immediately centrifuged and plasma stored at −20°C until analysis. Plasma concentrations of diflunisal were determined by HPLC based on a method developed by Hansen-Møller et al. (1987). To 100 µl of (diluted) plasma were added 200 µl of acetonitrile containing 4% acetic acid and naproxen as internal standard (5 µg/ml). After vortexing and centrifugation, a 20 µl aliquot of the supernatant was injected onto a 250 mm × 4 mm Lichrosorb RP-8 column (E. Merck, Darmstadt, Germany) with 4 µm particle size. The flow-rate of the mobile phase (methanol/20 mM potassium citrate buffer containing 0.02 mM tetramethylammonium sulfate, 57/43 v/v) was 0.8 ml/min. The eluate was monitored using a fluorescence detector (LS-40, Perkin Elmer, Beaconsfield, U.K.) at excitation and emission wavelengths of 315 and 389 nm, respectively. Using these conditions, diflunisal and the internal standard (naproxen) eluted from the column with retention times of approximately 7 and 9 min, respectively.

## 2.6. Data analysis

Results are expressed as mean number of flinches ±S.E.M. or mean licking time (sec) ±S.E.M. per observation period of 5 min. Multiple pairwise comparisons of the number of flinches or licking time, as a function of time following formalin injection, were made by analysis of variance (ANOVA) followed by the 'least significant differences' (LSD) test. A $P$-value smaller than 0.05 was considered significant.
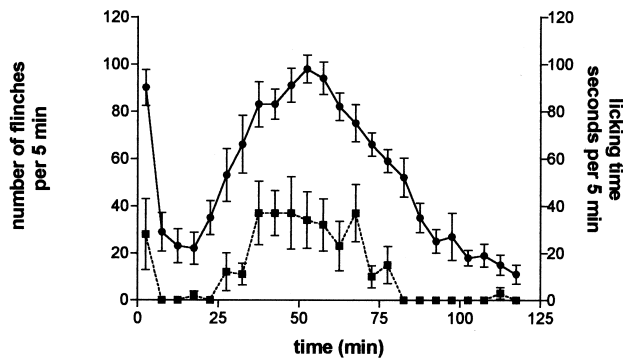
Fig. 1. The formalin test in rats. Formalin was injected at 10:00 a.m. in the right hind paw of 8 rats weighing 210–220 grams. Each point represents the mean ± S.E.M. number of flinches (●———●) or the mean ± S.E.M. time the rats spent licking/biting the injected paw (■- - -■) during a 5 min observation period.

## 3. Results

Two spontaneous behaviors indicative of pain were recorded following subcutaneous injection of formalin in the right hind paw: (1) flinching/shaking of the paw and/or hindquarters, and (2) licking/biting of the injected paw (Fig. 1). Both response curves exhibited a biphasic and parallel time course. The licking/biting behavior, however, showed a much higher intersubject variability (range of C.V.s at different time points: 73–265%) compared to the frequency of the flinching/shaking response (range of C.V.s at different time points: 17–105%). Because of the inherently greater intersubject variability in the licking/biting response, which was also observed in all subsequent experiments, the results of this report will mainly focus on the flinching/shaking nociceptive behavior.

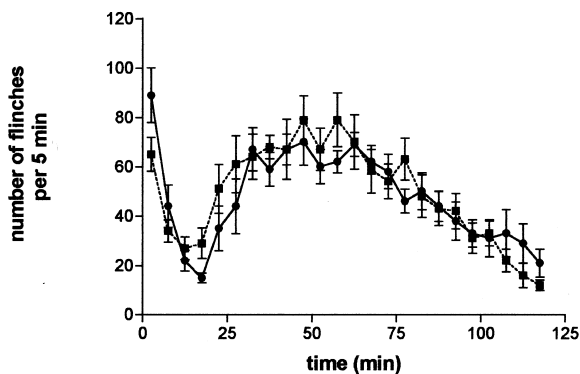The injection site, i.e right vs. left hind paw, did not affect the flinching/shaking behavior (Fig. 2) nor the

licking/biting response (data not shown). More importantly, this comparative study in two groups of 8 rats showed that reproducible results can be obtained despite a relatively important intersubject variability in the flinching/shaking behavior.

The age of the rat, as indicated by body weight, significantly affected the flinching/shaking behavior. Rats weighing 210–220 grams showed a significantly higher frequency of flinching/shaking during the second phase of this nociceptive response compared to rats weighing 240–250 and 270–280 grams (Fig. 3). No significant differences were found in the licking response to subcutaneous injection in the hind paw among these three groups of rats with different body weights (data not shown).

The time of day when the formalin test was carried out also significantly affected the nociceptive behavior of the rats. When formalin was injected in the morning (10:00 a.m.) the frequency of the flinching/shaking response was significantly higher during the second phase of the nociceptive behavior compared to a formalin test performed in the afternoon (2:00 p.m.) (Fig. 4). The licking/biting response to a formalin injection, however, did not significantly differ between the morning and the afternoon (data not shown).

The formalin test was used to evaluate the analgesic activity of diflunisal in rats weighing 210 to 220 grams. Formalin was injected into the right hind paw at 10:00 a.m., 30 min following a short (3 min) i.v. infusion of diflunisal at two different doses, i.e. 20 mg/kg and 100 mg/kg. Fig. 5 shows the mean diflunisal plasma concentrations during the formalin test. The major pharmacokinetic parameters of diflunisal obtained following i.v. infusion (3 min) of a 20 mg/kg and a 100 mg/kg dose are summarized in Table 1. Both the flinching/shaking and the licking/biting response were significantly affected by
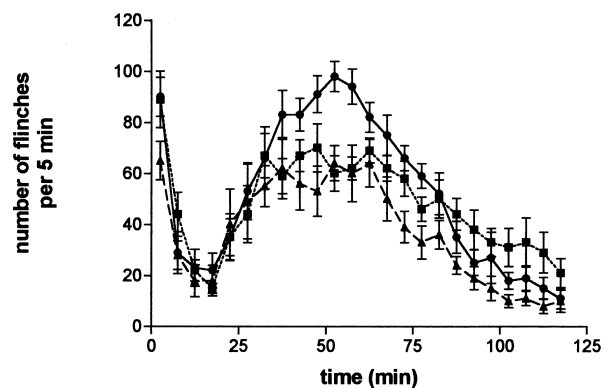


Fig. 2. The formalin test in rats: injection of formalin in the right vs. left hind leg. Formalin was injected at 10:00 a.m. in the right (●———●) or left (■- - -■) hind paw of rats weighing 210–220 grams. Each point represents the mean ± S.E.M. ($n=8$) number of flinches during a 5 min observation period. No statistically significant differences were observed between the two curves using ANOVA.



Fig. 3. The formalin test in rats: effect of age (body weight). Formalin was injected at 10:00 a.m. in the right hind paw of rats weighing 210–220 (●———●), 240–250 (■- - -■) or 270–280 grams (▲———▲). Each point represents the mean ± S.E.M. ($n=8$) number of flinches during a 5 min observation period. The intensity of the nociceptive behavior was significantly higher (ANOVA, LSD-test) in the 210–220 g rats compared to the 270–280 g rats during the following intervals: 0–5 min, 40–60 min and 65–80 min.
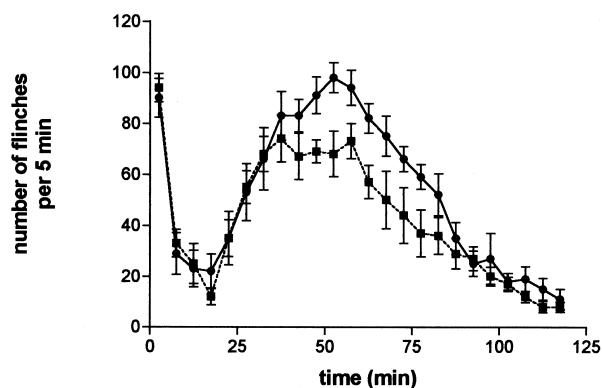
Fig. 4. The formalin test in rats: effect of time of day. Formalin was injected at 10:00 a.m. (●———●) or at 2:00 p.m. (■- - -■) in the right hind paw of rats weighing 210–220 grams. Each point represents the mean ± S.E.M. ($n=8$) number of flinches during a 5 min observation period. The intensity of the nociceptive behavior was significantly higher (ANOVA, LSD-test) for 45–80 min following formalin injection at 10:00 a.m.
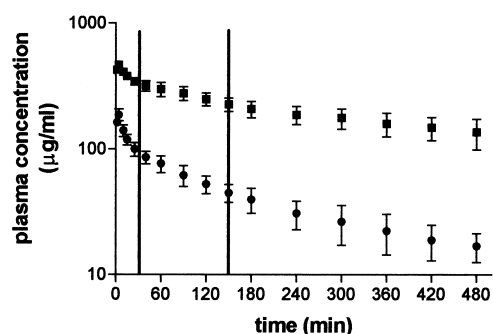


Fig. 5. Mean (± S.E.M., $n=6$) diflunisal plasma concentrations following i.v. infusion (3 min) of 20 mg/kg (●) or 100 mg/kg (■) diflunisal.
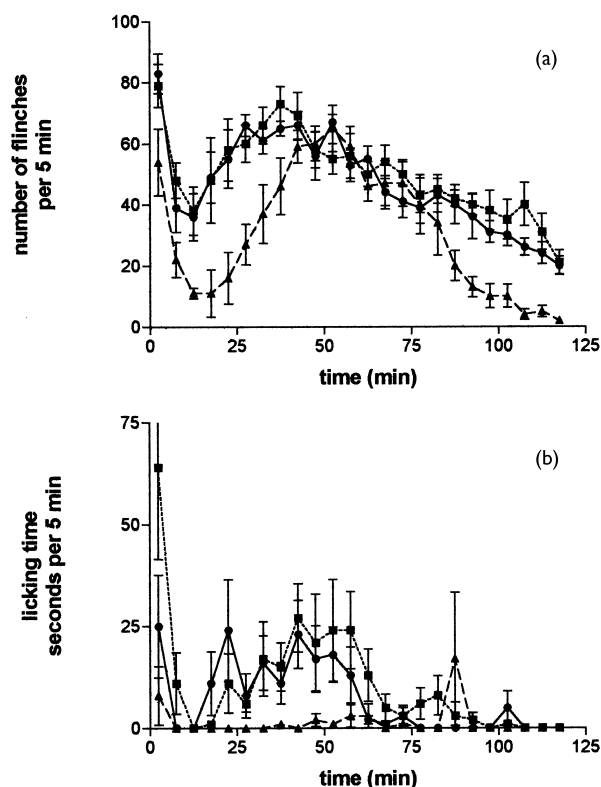


Fig. 6. The formalin test in rats: effect of diflunisal on the flinching/ shaking (A) and licking/biting (B) behavior. Formalin was injected at 10:00 a.m. (30 min following a 3 min i.v. infusion of diflunisal) in the right hind paw of rats weighing 210–220 grams. Each point represents the mean ± S.E.M. ($n=6$) number of flinches (panel A) or the mean ± S.E.M. ($n=6$) time the rats spent licking/biting the injected paw (panel B) during a 5 min observation period. Control (●———●), 20 mg/kg diflunisal (■- - -■), 100 mg/kg diflunisal (▲——— ——▲). The intensity of the nociceptive behavior following formalin injection was significantly reduced (ANOVA, LSD-test) by administration of 100 mg/kg diflunisal (3 min i.v. infusion) during the following intervals: 0–5 min, 10–40 min and 85–120 min for the flinching/shaking response (A) and 0–5 min, 20–55 min and 85–90 min for the licking/biting response (B).

diflunisal, but only at the 100 mg/kg dose level (Fig. 6). ANOVA showed a significant difference in the number of flinches vs. time curves between the 100 mg/kg i.v. treatment and control ($P<0.005$), whereas no significant difference was observed between the 20 mg/kg i.v. treatment and control rats ($P=0.181$). In addition, a significant delay was observed in the number of flinches vs. time profile following i.v. infusion (3 min) of 100 mg/kg diflunisal (Fig. 6A). The licking behavior was also significantly reduced following i.v. infusion (3 min) of 100 mg/kg diflunisal ($P<0.005$) but was not significantly affected by i.v. infusion (3 min) of a 20 mg/kg diflunisal

dose ($P=0.114$) (Fig. 6B). Control rats for these experiments were treated exactly the same way (surgical preparation, i.v. administration of vehicle 30 min before the formalin test, blood sampling before and during the test) as the diflunisal-treated rats. These control rats exhibited a different nociceptive behavior (both types) compared to control rats studied during the preliminary experiments who did not receive any treatment at all: between approximately 10 and 25 min following formalin injection their nociceptive response was significantly higher, whereas it was significantly reduced between approximately 35 and 75 min following formalin injection.

## 4. Discussion

The formalin test does not require sophisticated equipment and is very simple to carry out. Since the test

Table 1
Mean ± S.E.M. ($n=6$) values for the elimination half-life ($t_{1/2}$), plasma clearance (CL) and apparent distribution volume ($V_d$) of diflunisal following i.v. infusion (3 min) of a 20 mg/kg or a 100 mg/kg dose.

| Parameter | Diflunisal dose | |
|---|---|---|
| | 20 mg/kg | 100 mg/kg |
| $t_{1/2}$ (h) | 5.0±0.4 | 8.9±1.2 |
| CL (ml/min/kg) | 0.73±0.03 | 0.52±0.08 |
| $V_d$ (ml/kg) | 324±39 | 363±19 |

assesses the response of the animal to moderate, continuous pain, it has been proposed as a more valid model for clinical pain compared to tests using phasic thermal or mechanical stimuli. Because the test is based on the observation of spontaneous nociceptive behaviors following injection of formalin in the rat hind paw, intersubject variability is quite high especially for the licking/biting response. However, use of carefully conditioned animals and conduction of the test in a dedicated room under standard conditions such as time of day, room temperature (Rosland, 1991) and age (body weight) of the rats, will lead to reproducible results.

Nonsteroidal anti-inflammatory drugs (NSAIDS) have been previously shown to exhibit clear antinociceptive effects in the formalin test in the mouse (Hunskaar et al., 1986; Rosland et al., 1990) and in the rat (Malmberg and Yaksh, 1992). These studies demonstrated that NSAIDS such as indomethacin reduce nociceptive behavior during the second phase of the formalin test, while they do not seem to affect the first phase. Aspirin, on the contrary, showed a dose-dependent antinociceptive effect during both the early and the late phases (Hunskaar et al., 1986).

Results of the present study show that diflunisal, a salicylate analgesic, also had a clear effect on the second phase of the formalin test. Its effect on the first phase was less pronounced but nevertheless almost reached significance ($P = 0.059$) in case of the flinching/shaking behavior. This analgesic effect of diflunisal was dose-dependent: no effect was observed following i.v. administration of 20 mg/kg whereas a significant reduction in the nociceptive behavior (both flinching/shaking and licking/biting responses) was found following the 100 mg/kg dose.

Stone et al. (1977) studied the analgesic effect of diflunisal in the rat using a modification of the Randall-Sellito test (Randall and Sellito, 1957). One hind foot of the rat was rendered highly sensitive to pressure by injection of an irritant and the pressure, applied to the injected limb, was determined at which the rat would give a vocal response. They noted an analgesic effect of diflunisal at much lower doses, i.e. between 1 and 10 mg/kg. Similarly, Winter et al. (1979), using rats rendered hyperalgesic by injection of Freund's adjuvant in the tail, found an analgesic response in approximately 60% of the rats following oral administration of diflunisal at 20 mg/kg. Walker and Kasmerski (1988), using an electrical stimulus, studied the analgesic effect of diflunisal in control and arthritic rats. Analgesic effect of diflunisal was only studied and demonstrated following an 85 mg/kg i.v. dose.

Using the formalin test, a behavioral nociceptive test, we could not demonstrate an analgesic effect following a 20 mg/kg i.v. dose of diflunisal. Following a 100 mg/kg dose, however, a clear response was shown for both nociceptive behaviors. In case of the flinching/shaking behavior a significant inhibition of this nociceptive be-

havior was found until approximately 40 min following formalin injection. Between 40 min and 85 min following injection of formalin there was no difference between the treated and control rats. This temporary lack of effect of diflunisal on the flinching/shaking behavior cannot be explained by the fact that diflunisal concentrations continuously decreased during the observation period (see Fig. 5). Indeed, this decline in diflunisal plasma concentrations is rather small during the observation period, and, in addition, the licking/biting response was again significantly reduced from 90 min following formalin injection until the end of the observation period. Interestingly, the licking/biting response was almost completely abolished following 100 mg/kg diflunisal from 5 min following formalin injection onwards and lasts throughout the entire observation period.

In summary, the formalin test may be useful to study the analgesic effect of diflunisal in rats. It has, however, the following important drawbacks: (1) it requires a relatively large number of animals, and (2) it does not seem to be as sensitive to demonstrate an analgesic effect as other commonly used tests. On the other hand, this behavioral nociceptive test may be a more valid model for clinical pain (Dubuisson and Dennis, 1977; Abbott et al., 1981) and therefore deserves more attention.

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