Pharmacokinetic-Pharmacodynamic Modeling of the Antinociceptive Effect of Diclofenac in the Rat

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ABSTRACT

The relationship between the pharmacokinetics and the antinociceptive effect of diclofenac was evaluated using the pain-induced functional impairment model in the rat. Male Wistar rats were injected with uric acid in the knee joint of the right hind limb, which induced its dysfunction. Once the dysfunction was complete, animals received a p.o. dose of 0.56, 1, 1.8, 3.2, 5.6 or 10 mg/kg of sodium diclofenac, and the antinociceptive effect and drug blood concentration were simultaneously evaluated at selected times for a period of 6 h. Diclofenac produced a dose-dependent antinociceptive effect, measured as a recovery of the functionality of the injured limb. However, the onset of the antinociceptive effect was delayed with respect to blood concentrations. Moreover, the effect lasted longer than expected from pharmacokinetic data. Therefore, when functionality index was plotted against diclofenac blood concentration, an anticlockwise hysteresis loop was observed for all doses. Hysteresis collapse was achieved using the effect-compartment model, and the plot of functionality index against diclofenac concentration in the effect-compartment data was well fitted by the sigmoidal $E_{\text{max}}$ model. Our data suggest slow equilibrium kinetics between diclofenac concentration in blood and its site of action, which leads to a delayed onset of the antinociceptive effect as well as a longer duration of the response resulting from drug accumulation in synovial fluid.

Diclofenac is an NSAID that has been shown to be effective for relieving pain in rheumatic and nonrheumatic diseases (Menasse et al., 1978). The analgesic activity of diclofenac has been traditionally related to the inhibition of prostaglandin synthesis (Menasse et al., 1978). Other mechanisms, however, have also been suggested to be involved in the antinociceptive effect of this drug (Tonussi and Ferreira, 1978; Bjorkman, 1995).

On the other hand, it has been established that the relationship between pharmacokinetic properties and pharmacologic effect is the basis for a more rational drug regimen design, because it allows prediction of the time course of the intensity of the effect (Holford and Sheiner, 1981). This is one of the major goals in clinical pharmacology, but it is equally important in animal studies. For some drugs, a direct relationship between the effect and the drug concentration in an accessible body compartment, usually blood or plasma, has been found. In other cases, where the theoretical site of action is in a compartment not including blood or plasma, referred as the effect compartment, an indirect relationship between the pharmacologic effect and pharmacokinetics can be established (Holford and Sheiner, 1981).

There are reports wherein the anti-inflammatory and antinociceptive effect of diclofenac cannot be directly explained by circulating concentrations in animals (Menasse et al., 1978) or in humans (Todd and Sorkin, 1988; Ryhanen et al., 1994; Kurowski et al., 1994). It has been suggested that the antinociceptive and anti-inflammatory effects of diclofenac depend on the NSAID levels at the injured site, which may not be in equilibrium with the circulation (Kyuki, 1982). The purpose of this study was to perform pharmacokinetic-pharmacodynamic modeling for the antinociceptive effect of diclofenac, using an experimental pain model in the rat in order to understand the factors that determine the time course of diclofenac's effect.

Materials and Methods

Animals. Male Wistar rats (weighing, 180–220 g) from our own breeding facilities [Crl:(WI)BR], were used in this study. Animals were housed in a room with controlled temperature (22 ± 1°C) for at least 2 days before the study. Food was withheld for 12 h before the
initiation of experiments, but animals had free access to drinking water.

Surgery. The rats were lightly anesthetized with ethyl ether. Then PE catheters (a combination of PE-10 and PE-50 was used; I.D. 0.28 mm, O.D. 0.61 mm; I.D. 0.58 mm, O.D. 0.965 mm, respectively; Clay Adams, Parsippany, NJ) were surgically implanted into the caudal artery for the collection of blood samples as reported previously (Granados-Soto et al., 1995).

Chemicals. Sodium diclofenac was obtained from Ciba-Geigy (Mexico City, Mexico). Sodium naproxen was a gift of Syntex S.A. (Mexico City, Mexico). Uric acid was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and methanol were chromatographic grade (Merek, Darmstadt, Germany). Deionized water was obtained using a Milli-Q system (Continental Water Systems, El Paso, TX). Other reagents used in the study were of analytical grade.

Measurement of analgesic activity. All experiments followed the recommendations of The Committee for Research and Ethical Issues of the International Association for the Study of Pain (Covino et al., 1980) and The Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the study was approved by the local Animal Care Committee. Pain intensity and the antinociceptive effect of diclofenac were measured using the PIFIR model (López-Muñoz et al., 1993). Animals received an intra-articular injection of 0.05 ml of 30% uric acid suspended in mineral oil in the knee joint of the right hind limb under light anesthesia with ether. Then rats were cannulated in the caudal artery as described above, and an electrode was made to adhere to each hind paw behind the plantar pad. Rats were allowed to recover from anesthesia and were then placed on a stainless steel cylinder 30 cm in diameter rotating at 4 rpm and thus forcing the rats to walk. The variable measured in this model was the time of contact between each of the rat’s hind paws and the cylinder. When the electrode placed on the animal’s paw made contact with the cylinder floor, a circuit was closed and the time that the circuit remained closed was recorded. The cylinder was rotated for 2 min periods, during which time recordings were made; the rats were allowed to rest for 15 to 30 min between recording periods. During resting periods, rats did not show any sign of discomfort, such as licking, biting, shaking, elevating or vocalization, as in other pain models (Tjolsen et al., 1992). The PIFIR model allowed the animals freedom of choice. A nociceptive stimulus was produced by the pressure applied to the injured limb when the rat was walking. However, animals were able to avoid this noiception by walking with three limbs, i.e., avoiding the use of the injured limb.

After uric acid injection, rats developed a progressive dysfunction of the injured limb. This was recorded as a diminished time of contact between the right hind limb and the cylinder. Data are expressed as the FI, i.e., the time of contact of the injected limb divided by the time of contact of the control left limb multiplied by 100. After 2 h, FI was zero, i.e., the injured limb made no contact with the cylinder floor. At this time, rats received p.o. sodium diclofenac dose dissolved in saline solution (4 ml/kg), and recordings were made during the next 6 h. Recovery of FI was considered as the expression of the antinociceptive effect.

Analysis of diclofenac in blood. Blood concentrations of diclofenac were determined by a HPLC method developed in our laboratory. Briefly, whole-blood samples (100 μl) were placed into 1.5-ml Eppendorf tubes, and 50 ng of naproxen (internal standard) was added. Blood was thenacidified by the addition of 20 μl of 0.5 M NaOH (pH 2.5). Next 1 ml of ethyl acetate was added, and samples were extracted by agitation in vortex at maximal speed for 1 min. After centrifugation at 10,000 rpm for 10 min, the organic layer was transferred into a clean conical glass tube and evaporated to dryness in a water bath at 50°C under a gentle nitrogen stream. The dry residue was redissolved in 200 μl of a mixture of 0.075 M Na₂HPO₄ buffer (pH 7) and methanol (1:1), and 100-μl aliquots were injected into the chromatographic system.

The chromatographic system consisted of a model 510 solvent delivery system (Waters Assoc., Milford, MA), a 7125 Rheodyne injector with a 100-μl loop (Cotati, CA) and a LC-4B electrochemical detector (BAS, West Lafayette, IN) with a glassy carbon working electrode and an Ag/AgCl reference electrode. Compounds were separated at room temperature on a MicroPak C18 column of 300 mm × 4 mm I.D. and particle size of 10 μm (Varian, Palo Alto, CA) eluted with a mixture of 0.075 M sodium acetate (adjusted to pH 3.3 with glacial acetic acid) and acetonitrile (55:45, v/v) at a flow rate of 2 ml/min. The detector was operated at +1.1 V, and the chromatograms were registered in a Servogor 120 (Norma Goerz Instruments, Elik Grove Village, IL). Retention times were 3.5 and 6 min for naproxen and diclofenac, respectively. Calibration curves were constructed for diclofenac concentrations in blood ranging from 25 to 2000 ng/ml. A linear relationship (r = 0.9996) was obtained when peak-height ratios of diclofenac to the internal standard were plotted against diclofenac blood concentration. Coefficients of variation were always lower than 10%, whereas accuracy ranged from 90% to 115%. The detection limit of the method was 10 ng/ml.

Study design. In this study, the antinociceptive effect of diclofenac and its circulating concentrations were estimated simultaneously in the same animal, following a design similar to that previously reported for the pharmacokinetic-pharmacodynamic analysis of ketorolac (Granados-Soto et al., 1995). Five groups of six rats each were used in this study. Each group received an oral dose of 0.56, 1, 1.8, 3.2, 5.6 or 10 mg/kg sodium diclofenac. FI was measured at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 and 6 h after dosing. Immediately after FI determination, blood samples (100 μl) were obtained through the cannula inserted into the caudal artery. Blood samples were frozen at −70°C until analyzed for diclofenac by HPLC.

Two additional control groups were studied. Animals in the first control group received an intra-articular injection of mineral oil without uric acid. Animals in the second control group were injured with intra-articular uric acid but did not receive any antinociceptive agent.

Analysis of results. Maximal diclofenac blood concentrations (Cmax) were determined directly from individual concentration-time curves. AUC to the last measurable point was calculated by the trapezoidal rule (Rowland and Tozer, 1989). Emax, str were determined directly from individual FI-time curves. AUCR, a global expression of the antinociceptive effect of diclofenac, was determined by the trapezoidal rule.

Mean blood concentration-time data were fitted to the two-open compartmental model (Gabrielsson and Weiner, 1994), according to equation 1.

\[
C = \frac{KAD \times (K21 - \alpha)e^{-\beta t}}{Vd/F \times (\alpha - \beta) + (\alpha - \beta)(e^{-\alpha t} - e^{-\beta t}) + (\alpha - KA)e^{-\beta t}}
\]  

where C is the diclofenac blood concentration, KA is the absorption rate constant, K is the elimination rate constant, K21 is the transferance rate constant from the peripheral to the central compartment, Vd/F is the volume of distribution corrected by the bioavailability of the oral dose D and α and β are the hybrid rate constants corresponding to the initial and terminal slope factors, respectively. The antinociceptive effect of diclofenac, expressed as FI recovery, was plotted as a function of drug concentration in blood. If the resulting curve exhibited a counterclockwise hysteresis loop, then an equilibrium delay between the central and effect compartments was suggested. A pharmacokinetic model linked to an effect compartment was used to collapse the hysteresis loop as described by Holford and...

$$Ce = \frac{KAD}{\text{VdTF}} \left( \frac{(K21 - KA)e^{-KAt}}{(\alpha - KA)(\beta - KA)(Ke0 - KA)} + \frac{(K21 - \alpha)e^{-\alpha t}}{(\alpha - \alpha)(\beta - \alpha)(Ke0 - \alpha)} + \frac{(K21 - \beta)e^{-\beta t}}{(\alpha - \beta)(\beta - \beta)(Ke0 - \beta)} + \frac{(K21 - Ke0)e^{-Ke0t}}{(\alpha - Ke0)(\beta - Ke0)} \right)$$

(2)

where $Ce$ is the effect-compartment concentration and $Ke0$ is the constant of the disappearance of the effect. Other pharmacokinetic parameters have been defined above.

$FI$ and $Ce$ were related using the sigmoidal $E_{\text{max}}$ model (Holford and Sheiner, 1981) according to equation 3.

$$E = \frac{E_{\text{max}} \cdot Ce^h}{EC_{50}^h + Ce^h}$$

(3)

where $E$ is the observed effect, $E_{\text{max}}$ is the theoretical maximal effect that can be attained, $Ce$ is the effect-compartment concentration, $EC_{50}$ is the $Ce$ value that produces an effect equivalent to 50% of the theoretical maximal effect and $h$ is a parameter that determines the steepness of the curve.

All fitting procedures were performed by a nonlinear regression routine using the PCNONLIN software (Metzler and Weiner, 1992). A combination of the pharmacokinetic and pharmacodynamic models was used to describe the intensity of the effect as a function of time. Fittings were carried out as described by Gabrieleson and Weiner (1994). Initially, we performed a pharmacokinetic fitting, taking into account the data derived from all the doses assayed. A weight factor of $1/C^2$ was considered. The obtained pharmacokinetic parameters were then used to estimate effect-compartment concentrations, and the relationship between these concentrations and the observed antinociceptive effect was determined. Pharmacokinetic-pharmacodynamic fittings also included data from all doses, but no weighing scheme was used in this case.

Results

The measurement of nociception and of antinociceptive effect using the PIFIR model is shown in figure 1. Rats that were injected with mineral oil without uric acid exhibited FI values of 100%; i.e., the times of contact of both hind limbs when walking were similar. Uric acid injection produced a progressive dysfunction of the injured limb, observed as a reduction in FI. Values reached zero 2 h after uric acid injection. If no analgesic agent was given, there was no spontaneous recovery of FI during the 6-h observation period. Animals that received diclofenac 2 h after uric acid injection exhibited a gradual recovery of FI.

The time courses of diclofenac blood concentration and of FI observed with six doses studied are shown in figure 2. Diclofenac blood levels increased very rapidly, whereas FI values increased gradually. The bioavailability parameters $C_{\text{max}}$ and $AUC$ increased with the diclofenac dose, which suggests linear pharmacokinetics (table 1). The pharmacodynamic parameters $E_{\text{max}}$ and $AUC$ also increased with the dose. Notwithstanding, it appeared that saturation of the effect was reached, because all doses above 3.2 mg/kg exhibited a similar FI-time profile (fig. 2; table 1).

As a consequence of the different time courses of blood
concentration and antinociceptive effect, when FI recovery was plotted against blood concentration, the resulting curves exhibited an anticlockwise hysteresis loop (fig. 3); this was observed with all the doses studied. Assuming that the effect was related to diclofenac concentration in an effect compartment, we performed pharmacokinetic-pharmacodynamic modeling. Fittings were carried out including data from all the doses assayed. Initially, data on mean blood concentration against time were fitted to the open two-compartment model by equation 1. Then hysteresis collapse was achieved, using equation 2, by assuming that the effect depends on diclofenac concentration in an effect compartment rather than in the circulation. Finally, the observed FI recovery was related to Ce by the sigmoidal \( E_{\text{max}} \) pharmacodynamic model, using equation 3. Dose-independent pharmacokinetic and pharmacodynamic parameters obtained with these fittings are listed in table 2. As figure 4 shows, the effect data derived from all the doses studied were well described as a function of the estimated Ce values.

**Discussion**

There are few reports about the relationship between the pharmacokinetics and the antinociceptive effect for either opioids or nonsteroidal anti-inflammatory drugs in either clinical or animal models. This is probably because of the scarcity of suitable pharmacological models that allow quantitative evaluation of the time course of the antinociceptive effect in animals or humans (Dunagan et al., 1986). As we have previously reported, the PIFIR model seems to be an
adequate model for performing pharmacokinetic-pharmacodynamic studies, because one can use it to determine the intensity of the antinociceptive effect at different times, while respecting the ethical standards for the study of pain in experimental animals (López-Muñoz et al., 1993; Granados-Soto et al., 1992, 1995).

In this study, the PIFIR model was used to carry out a pharmacokinetic-pharmacodynamic evaluation of diclofenac. Diclofenac administered p.o. produced an antinociceptive effect in a dose-dependent manner. This effect was of slow onset, however, whereas diclofenac circulating concentrations increased rapidly, reaching maximal blood levels in about 0.3 h. Moreover, diclofenac concentrations decreased after the peak, while the antinociceptive effect was still rising. Hence it appears that the antinociceptive effect of diclofenac in this model cannot be explained by its circulating concentrations. These results are consistent with those reported by Menassé and co-workers (1978), who observed that the anti-inflammatory effect of diclofenac in an experimental model of inflammation lasted for several hours even if the drug was no longer detectable in the circulation. Results that suggest a delay in the appearance of the antinociceptive or anti-inflammatory effect of diclofenac with respect to circulating drug levels have also been observed in humans (Todd and Sorkin, 1988; Ryhanen et al., 1994; Kurowski et al., 1994).

The time course of the antinociceptive effect of diclofenac was different from that reported for acetaminophen (Granados-Soto et al., 1992) and ketorolac (Granados-Soto et al., 1995) in the PIFIR model. For these two drugs, the antinociceptive effect exhibited a fast onset, and it was possible to relate it directly to circulating drug concentration. On the other hand, when the antinociceptive effect, expressed as FI recovery, was plotted as a function of diclofenac blood levels, the resulting curve exhibited an anticlockwise hysteresis loop, which indicates the lack of a direct relationship (Holford and Sheiner, 1981). Several explanations have been proposed for such plots, including the formation of active metabolites, an effect compartment different from those detected by conventional pharmacokinetic analysis (Holford and Sheiner, 1981) and a cascade of physiological events (Dayneda et al., 1993). The possibility of active metabolites can be discarded, because it has been shown that local administration of diclofenac results in an anti-inflammatory effect (Kyuki, 1982; Tonussi and Ferreira, 1994), whereas the known diclofenac metabolites are devoid of any antinociceptive activity (Menassé et al., 1978; Faigle et al., 1988). Our data appear to favor the hypothesis of the different effect compartment, because effect-compartment concentrations calculated by considering a fixed Ke0 value were able to account for the antinociceptive effect observed with all the doses studied according to the same Hill equation. It is possible to conceive that the time lag between circulating concentrations and the antinociceptive effect is due to a cascade of physiological events, because diclofenac's antinociceptive effect is an indirect response resulting from inhibition of prostaglandin synthesis and from other mechanisms of action (Garg and Jusko, 1994). However, this does not appear to be the case. If the delay were due to a slow sequential activation of physiological events, then dose-dependent changes in Ke0 as well as in the parameters of the Hill equation should be observed (Dayneda et al., 1993). Moreover, there is evidence that diclofenac has a rapid effect when administered locally (Kyuki, 1982; Tonussi and Ferreira, 1994). These results strongly suggest that the sequence of events leading to the antinociceptive effect of diclofenac unfolds rapidly once the drug reaches its site of action and thus cannot account for the delayed onset of response after systemic administration. Hence the lag in the onset of the antinociceptive effect relative to the drug’s appearance in the circulation, as well as its longer duration than that expected from pharmacokinetic data, can reasonably be explained by slow equilibrium kinetics between diclofenac concentration in the central and effect compartments.

The PIFIR is an inflammatory model of noceception, because uric acid injection in the knee causes articular inflammation in a manner similar to gout (López-Muñoz et al., 1993). It has been suggested that synovial fluid is the main site of action of NSAIDs in arthropathy (Netter et al., 1989). In the case of diclofenac, there is evidence that this agent is transferred across the synovial membrane to the synovial fluid, from which is eliminated more gradually than from plasma (Fowler et al., 1983, 1986; Radermacher et al., 1991). It has been suggested that the clearance of diclofenac from synovial fluid to blood occurs slowly because the drug binds with high affinity to the albumin that is sequestered in the synovial space in arthropathy (Owen et al., 1994). Therefore, the prolonged antinociceptive effect of diclofenac may be explained by the fact that the drug is retained by the albumin-enriched synovial fluid. It then appears that the explanation of a delayed antinociceptive action of diclofenac in inflammatory pain is supported not only by pharmacokinetic-pharmacodynamic analysis, as in the results here presented, but also by the information available on the physiological action of this drug.

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