

PK/PD evaluations of the novel atypical opioid tapentadol in red-eared slider turtles^{*)}

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Summary

The aim of this study was to evaluate the pharmacokinetics and pharmacodynamics of tapentadol (TAP) in red-eared slider turtles after a single intramuscular injection of 5 mg/kg. Turtles (n = 9) were randomly assigned to two treatment groups, according to an open, single-dose, single-treatment, unpaired, two-period crossover design. Group A (n = 5) received a single IM dose of TAP (5 mg/mL) at 5 mg/kg. Group B (n = 4) received a single IM injection of saline (equivalent to the opioid in volume). After a one-month wash-out period, the groups were rotated, and the experiment was repeated. TAP plasma concentrations were evaluated by a validated HPLC-FL method, while an infrared thermal stimulus was applied to the plantar surface of the turtles' hind limbs to evaluate the thermal withdrawal latency (TWL). TAP plasma concentrations were detectable between 1 and 24 h (2141 – 42 ng/mL, respectively). The TAP-treated group showed a dramatic increase in TWL one hour after drug administration (15.31 ± 4.73 s). Subsequently, TWL decreased with time. Significant differences between the treatment and control groups were apparent up to 10 h following treatment. A linear relationship ($r^2 = 0.98$) between the TAP plasma concentration and effect was found. Given these qualities, TAP appears to be an attractive option for antinociception in turtles because of its rapid onset and acceptable duration of effect.

Keywords: opioids, pharmacokinetics, tapentadol, thermal withdrawal latency, turtles

Veterinary medicine faces the unique challenge of having to treat many animal species, including mammals, birds, reptiles and fish. The main challenge for veterinarians is not just to select a drug, but also to determine, a rational dosing regimen for the selected agent. Determining this is a long and complicated endeavour because of differences in the expression of enzymes, receptors and signal transduction molecules between species (9). Both inter- and intra-species differences in drug response can be accounted for as either being due to variations in drug pharmacokinetics (PK) or drug pharmacodynamics (PD), the magnitude of which varies from drug to drug (26). Hence, PK/PD studies are critical when a drug is applied to a new animal species.

Nowadays we are far more cognisant of pain in animals. Animal species that one decade ago were considered wild animals are now pets, and their owners

expect an adequate level of care to be provided. This change in attitude has resulted in a push for the development of more effective and innovative veterinary therapies (14, 16, 18). With the increasing popularity of herpetoculture, there is more information on associated diseases, and treatment options are being investigated, starting with the classes of drugs that have proven efficacy in other species. This research has emphasized the inaccuracies that result when the effects and consequences of drugs for the species of interest are predicted by extrapolation from other species that are markedly different in their physiology (21).

Opioids are considered the most effective drugs for controlling pain in mammals (4). Veterinary clinicians have a number of options: the classical μ -opioid receptor (MOR) agonists (eg, morphine), the partial MOR agonists (eg, buprenorphine), the mixed opioid κ -receptor agonists MOR-antagonists (eg, butorphanol), and the atypical opioids (eg, tramadol).

In reptiles, opioid drug administration has yielded unexpected results with respect to analgesia. Butor-

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phanol does not change thermal withdrawal latencies (TWL) in red-eared slider turtles (30) and bearded dragons (28) or thermal thresholds in green iguanas (6). Buprenorphine did not alter responses to a noxious electrical stimulus administered to green iguanas (19), and did not provide an analgesic effect in red-eared slider turtles exposed to a noxious thermal stimulus (23). Morphine increased TWL in turtles (30) and bearded dragons (28) at doses ranging between 1.5 and 20 mg/kg, but was ineffective at doses up to 40 mg/kg in corn snakes (28). In contrast, the atypical opioid tramadol, whose use in mammals has been widely questioned (10, 15, 17), has proven to be effective (10 mg/kg SC) for at least 48 hours following administration in turtles (1). Tramadol produces MOR activation (6000 times lower than morphine), as well as the inhibition of serotonin (5HT) and noradrenalin (NA) reuptake. It has been shown that the analgesic efficacy of tramadol is mediated by the M1 metabolite (200-300 times more potent on MOR activation than the parental compound) (25).

Tapentadol (TAP) is a novel atypical opioid drug labelled for human use. Because of its unique mechanism of action, it has been proposed as the first representative of a new pharmacological class of centrally acting analgesics: the MOR agonist, NA reuptake inhibitors (8). Interestingly, even though its MOR affinity is 50 times lower than that of morphine, it is only 2-3 times lower after systemic administration. This finding, consistent across different pain relief evaluation models, may be due to a better brain penetration of TAP, but it also suggests that the NA reuptake-inhibitory property contributes to a more potent analgesia that would be expected solely from its MOR agonism (34). Given the moderate affinity of TAP at the MOR and the opioid-sparing effect of TAP's NRI component, it seems logical that TAP would produce fewer opioid-related side effects than classical MOR agonists, such as morphine. Hence, the aim of this study is to assess the PK/PD of TAP in red-eared slider turtles after a single intramuscular injection of TAP.

Material and methods

Material. TAP hydrochloride was supplied as a pure powder (> 99.8% purity; Bepham). M1, the metabolite of tramadol, was used as an internal standard and supplied as pure powder (> 99.8% purity; LCG Promochem). Additionally, high-performance liquid chromatography (HPLC) grade acetonitrile (ACN), dichloromethane (CH₂Cl₂) and diethyl ether (Et₂O) were used in the assays (Scharlau). Acetic acid and sodium tetraborate decahydrate (BDH) were of analytical grade. HPLC grade water was obtained by distilling deionised water produced by a Milli-Q Millipore water system (EDM Millipore). All the other reagents and materials were of analytical grade and supplied from commercial sources.

Animals and experimental design. Nine turtles (*Trachemys scripta elegant*) of undetermined sex, with body weights ranging from 0.8 to 1.5 kg (mean 1.1 kg), sup-

plied by a local park, were used for the study. Turtles were acclimated for a 2-week period prior to the study. Turtles were judged to be in good health on the basis of physical examination at the time of acquisition and at the start of the study, and through daily observation of behaviour and appetite. These observations were made by specialized veterinary personnel. Turtles were divided into groups A or B, and each group was placed in a separate 300 L plastic pool, with a water depth of 20 cm, water temperature of 27°C, and custom-built mechanical and biological filtration. A dry basking area was heated to 30°C with an infrared lamp. The ambient temperature in the room varied from 25 to 26°C (electronic temperature sensors assured constant temperature in the water and the basking area). Turtles were fed a floating pelleted diet three times a week. Animal care and handling were performed according to the provision of the EC council Directive 86/609 EEC and also according to Institutional Animal Care and Use directives issued by the Animal Welfare Committee of the University of Pisa, which approved the study protocol.

Turtles were randomly assigned to two treatment groups, by means of slips of paper marked with the numbers 1-9, selected blind from a box. An open, single-dose, single-treatment, unpaired, two-period crossover design was used. Each turtle in group A (n = 5) received a single IM dose of TAP at 5 mg/kg (5 mg/mL). This dose was selected on the basis of previous information describing the effectiveness of TAP in rabbits (13). Group B (n = 4) received a single IM injection of saline (equivalent to TAP solution in volume). The injectable TAP solutions were prepared by dissolving pure TAP hydrochloride powder in saline to produce a 5 mg/mL solution, which was then passed through a 0.45 µm filter under sterile conditions. A one-month wash-out period was observed to ensure a complete metabolism and excretion of TAP. After this period, the groups were rotated, and the experiment was repeated (second period). By the end of the study, each turtle had received both the saline and TAP treatments. Blood samples (1 mL) were collected from the dorsal cervical, subcarapacial venipuncture site at 0, 1, 2, 4, 6, 10, and 24 h after TAP administration and placed in collection tubes containing lithium heparin. Specimens were centrifuged at 1,000 g within 30 min of collection, and the harvested plasma was stored at -70°C and used within 15 days of collection.

Thermal analgesia experiments. Just before each blood collection, analgesia experiments were conducted by applying infrared thermal stimuli to the plantar surface of the turtles' hind limbs with a plantar analgesia device (Hargreaves's instrument, model 37370, Ugo Basile) according to previously described methods (1, 28, 30) with slight modifications. Turtles were gently dried with a smooth cloth and individually placed in clear, plastic boxes (300, 200, 150 mm, W, L, H, respectively, with a 1 mm thickness) on a clear acrylic surface. The room temperature was set at 25-26°C. An infrared radiation source was activated (70°C) directly below the surface on which the turtle rested the plantar surface of either hind limb. Hind limb TWLs were measured by a motion-sensitive timer, which stopped automatically when the hind limb was removed from the noxious stimulus. The increasing temperature caused the turtle to withdraw the limb, and the time to withdrawal was

automatically measured. A maximum exposure duration of 22.5 s (cut-off time) was allowed to prevent tissue damage. At each time point, the TWL was measured in both hind limbs. When the difference between the two TWL values was > 2 s, a third measurement was obtained. The observer in the analgesia experiments was blinded to treatments received. TWLs were measured before drug administration (baseline) and at the time of blood collection.

The thermal antinociceptive effect was expressed as percentage of Maximum Possible Response (% MPR) (20), which was calculated as:

$$\%MPR = \frac{T_{test} - T_{con}}{T_{cut} - T_{con}} \times 100\%$$

where T_{test} represents TWL value after injection of TAP, T_{con} is TWL value after injection of saline (control) and T_{cut} is the cut-off time (22.5 s).

High performance liquid chromatography (HPLC). Based on a previously published HPLC technique (12), the analytical method was re-validated for turtle plasma samples. The intra- and inter-day repeatability was measured as a coefficient of variation and was lower than 10.2%, whereas accuracy, measured as closeness to the concentration added on the same replicates, was lower than 5.9%. The limits of detection (LOD) and quantification (LOQ) were 0.5 ng/mL and 2 ng/mL, respectively. The chromatographic separation assay was performed with a SunFire C18 analytical column (150 × 4.6 mm inner diameter, 5 μm particle size, Water), maintained at 25°C. The mobile phase consisted of ACN (A): 0.2% acetic acid (B) at a flow rate of 1 mL/min. Excitation and emission wavelengths were set at 273 and 298 nm, respectively. The linear gradient elution system was performed as follows: 5-95% B (0-20 min), 95-5% B (20-25 min) and 5% B isocratically (25-32 min).

Preparation of plasma samples. Briefly, 50 μL of IS solution (0.5 μg/mL) and 0.2 mL 2 mM borate buffer, adjusted to pH 9.3, were added to a 1.5 mL polypropylene snap cap tube (Sarsedt) containing 0.5 mL of plasma. After vortex-mixing, 0.4 mL of extraction solvent (Et₂O : CH₂Cl₂ 7 : 3 v/v) was added. The tube was then placed in a vortex for 30 s, shaken for 5 min, and then centrifuged for 10 min at 15,625 g. The organic layer (0.3 mL) was then transferred into a clean 1.5 mL polypropylene snap cap conical tube, placed in a vortex and then shaken with 0.2 mL of back-extraction solvent (0.05 M HCl : ACN 1 : 1 v/v) for 5 min, before being centrifuged for 10 min at 15,625 g. The aqueous phase (50 μL) was injected into the HPLC system.

Pharmacokinetic evaluation. The pharmacokinetic calculations were carried out by WinNonLin v 5.3 (Pharsight). The maximum concentration (C_{max}) of TAP in plasma and the time required to reach C_{max} (T_{max}) were predicted from the data. The terminal rate constant (λ) was determined from the slope of the terminal phase of the plasma concentration curve that included a minimum of three points. The half-life of the terminal phase ($T_{1/2\lambda}$) was calculated with $T_{1/2} = 0.693/\lambda$. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. Changes in the plasma concentration of TAP were evaluated by the standard non-compartmental analysis, and the relative pharmacokinetic parameters were determined using standard non-compartmental equations (7).

Statistical analysis. The Kolmogorov-Smirnov test was applied to verify data distribution. Pharmacodynamic data were evaluated by Student's t test to determine statistically significant differences. The TAP plasma concentrations and the pharmacokinetic and pharmacodynamic parameters are presented as means ± standard error mean. All analyses were performed by GraphPad InStat (GraphPad Software). In all experiments, differences were considered significant if $P < 0.05$.

Results and discussion

One hour after the TAP IM administration, some signs of sedation were noticed in the animals. The turtles did not appear to be responsive to external stimuli (eg, drying process) and had flaccid limbs and necks compared to the control animals. This effect was transient and was almost completely absent at 2 h following drug administration. Unfortunately, the respiratory rate was not evaluated because of the lack of a breathing chamber described earlier (30).

Tab. 1. Pharmacokinetic parameters after IM injection of TAP at 5 mg/kg in turtles (n = 9)

Pharmacokinetic parameters	Unit	Mean ± SE
λ	1/h	0.29 ± 0.19
$T_{1/2\lambda}$	h	5.22 ± 2.98
T_{max}	h	1.42 ± 0.39
C_{max}	ng/mL	1899 ± 242
$AUC_{0-\infty}$	h ng/mL	8987 ± 4879
VZ/F	L/kg	4.65 ± 1.34
CL/F	L/min/kg	0.92 ± 0.66
MRT	h	5.74 ± 1.39

Explanations: λ – first-order rate constant; $T_{1/2\lambda}$ – half-life of the terminal portion of the curve; T_{max} – time at the maximum drug concentration; C_{max} – maximum drug plasma concentration; $AUC_{0-\infty}$ – area under the curve from 0 to infinity; VZ/F – apparent volume of distribution; CL/F – apparent total body clearance; MRT – mean residence time

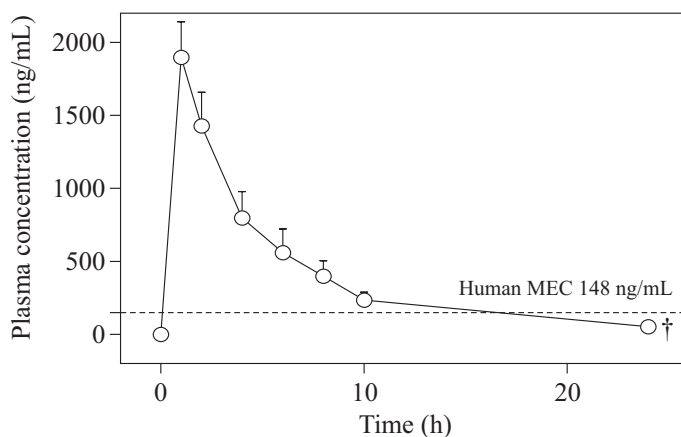


Fig. 1. Mean plasma concentrations of TAP (± SE) vs. time curve after IM administration in turtles (n = 9). The dashed line shows the Minimal Effective Concentration (MEC) of TAP reported for humans (148 ng/mL). † Data obtained in seven turtles

Pharmacokinetics (PK). The average TAP plasma concentration vs. time curve after IM administration of 5 mg/kg in turtles is presented in Fig. 1. The absolute plasma concentrations of TAP were in the range of 42-2141 ng/mL and detectable up to 24 h, except in two subjects. The corresponding pharmacokinetic parameters are shown in Table 1. The theoretical peak plasma drug concentration (C_{max}) of 1899 ± 242 ng/mL was observed at 1.42 ± 0.39 h (T_{max}) after injection. TAP was eliminated slowly in turtles with a long terminal half-life of 5.22 ± 2.98 h, and it showed a large volume of distribution (VZ/F) of 4.65 ± 1.34 L/kg.

Pharmacodynamics (PD). Differences in TWL in the control group animals (n = 9) were statistically insignificant at any point tested. Hence, to establish the TWL base line, all the saline solution data were grouped for each time point. The T0 was 4.9 ± 0.50 s with average values of the whole base line ranging from 4.90 to 5.56 s (Fig. 2A).

The animals given TAP showed a drastic increase in TWL one hour after drug administration (15.31 ± 4.73 s). Subsequently, TWL decreased in proportion to time with significant differences from the saline group still

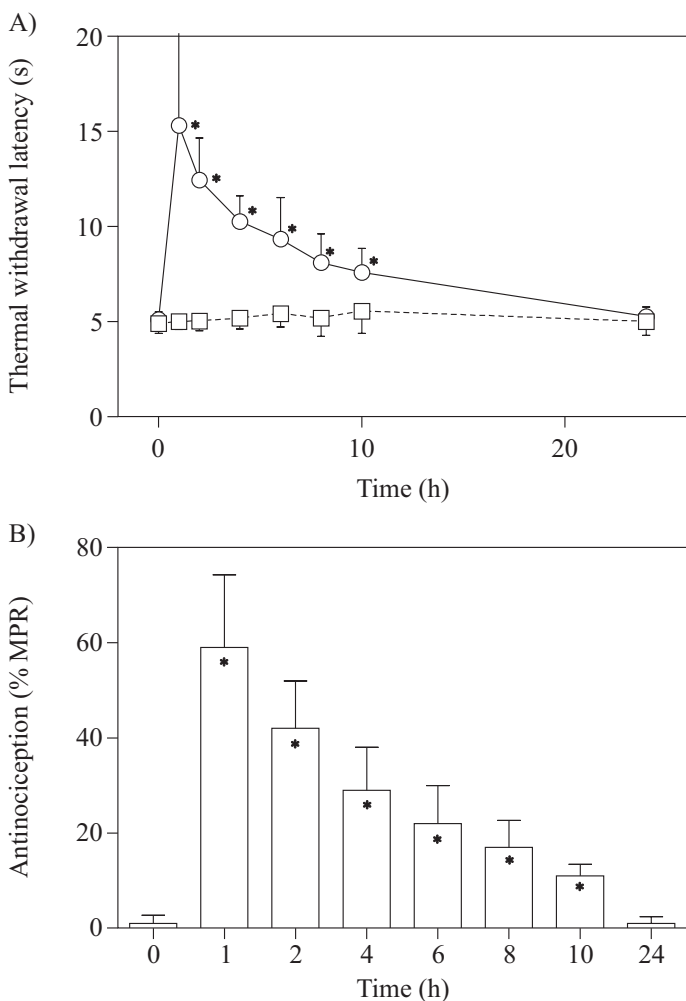


Fig. 2. (A) Mean (\pm SE) TWL vs. time curve in turtles (n = 9) after IM saline (open square) and IM TAP (open circle) administration (5 mg/kg); (B) mean (\pm SE) % MPR after IM administration of TAP (5 mg/kg). * Significantly different ($P < 0.05$) from saline value (control)

apparent up to 10 h. The average TWL value in the TAP group after 24 h was 5.01 ± 0.73 s, which is still greater than that of baseline, but not significantly so ($P = 0.18$).

Mean MPR started at $1.31 \pm 1.98\%$ (T0), increased to a maximum of $59.01 \pm 15.30\%$ at 1 h and decreased to a minimum of $1.00 \pm 1.51\%$ at 24 h (Fig. 2B). The MPR difference between the TAP and saline groups was still significant at 10 h.

PK/PD evaluation. The pharmacokinetic/pharmacodynamic correlations are reported in Fig. 3A, B. The mean TAP plasma concentration and % MPR vs. time curves were very similar (Fig. 3a). The average plasma concentration associated with a maximum % MPR of $59.01 \pm 15.30\%$ was 1899 ± 242 ng/mL. The mean plasma concentration at each time point ranged between 52.34 ng/mL (24 h) and 1619 ng/mL (1 h), associated with % MPR of 1.00 and 59.01%, respectively. A linear relationship ($r^2 = 0.98$) between TAP plasma concentration and % MPR was found (Fig. 3B).

If it is difficult to recognize whether an animal feels pain, it is even more challenging to determine objectively whether pain medication is effective in exotic animals. In general, to determine the efficacy of drugs in

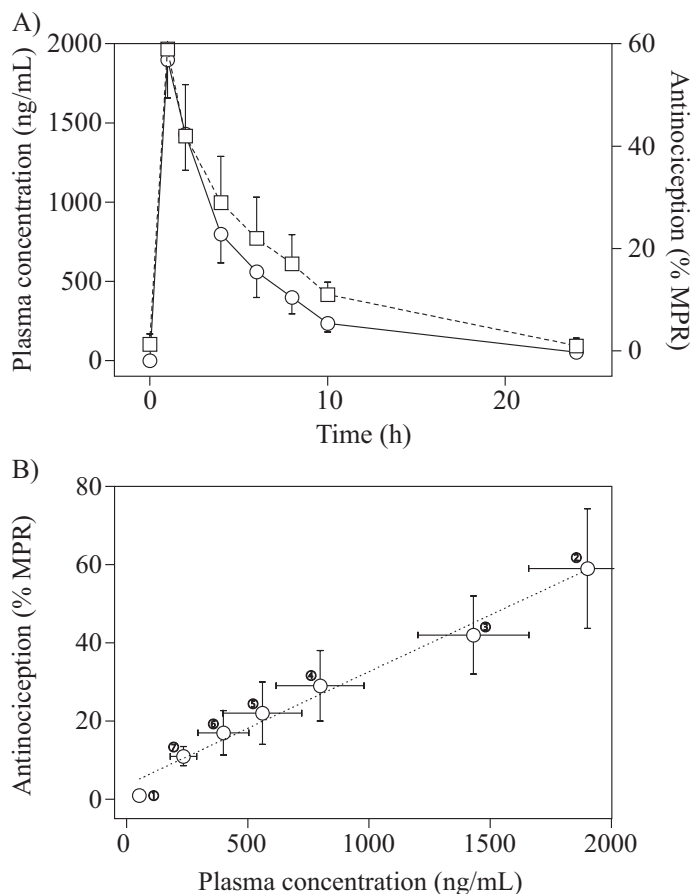


Fig. 3. (A) Mean (\pm SE) experimental plasma concentrations (open circles) of TAP and mean (\pm SE) % MPR (open squares) vs. time curves in turtles (n = 9) after IM TAP administration (5 mg/kg); (B) mean (\pm SE) experimental plasma concentrations (open circles) vs. mean (\pm SE) % MPR curve. The dotted line is the computed correlation line (experimental plasma concentrations vs. % MPR). Numbers in the circles represent time order

any species, it is important to determine the pharmacokinetic and pharmacodynamic properties of the drug in that species (32). Knowing pharmacokinetic values for a specific analgesic is often insufficient to determine appropriate doses and dosing frequencies, because plasma levels of drugs do not always correlate with analgesia. Plasma concentrations can provide guidance for dosing frequencies, but that does not always hold true, because the effect of analgesics (eg. NSAID) may be much longer than what would be expected from plasma levels. The pharmacokinetics of analgesics also vary considerably across all species that have been studied, so extrapolating clinical doses and dosing intervals from one species to another is not appropriate (9).

There is great potential for the use of TAP in veterinary species (8). Its PK profiles have already been tested in dogs (11), cats (22) and goats (Prof. E. Lavy, unpublished data), and its PK/PD profile has been assessed in rabbits (13). Although TAP is an atypical opioid, like tramadol, it might theoretically be free of some disadvantages of tramadol reported in mammals, because [1] TAP exists only as a single enantiomer (including both mechanisms of action in the same molecule); [2] only the parent compound is involved in its pharmacological activity (ie, no metabolic activation is necessary); [3] time-dependent changes in the dynamics of opioid and monoaminergic analgesia occur in parallel; [4] there is no CYP450 induction/inhibition that could negatively affect analgesia; [5] TAP is likely to produce less side effects compared with classical opioids such as morphine because of its lower MOR affinity (34). However, reptiles seem to react to opioids differently than mammals, and therefore a PK/PD study in turtles is essential to understand the effectiveness of this drug.

Several nociceptive tests have been established for use in vertebrates, but only a few are available for reptiles. In the present study, the TWL was evaluated using a noxious heat radiant model with an automatic motion sensor device. This method is easy, fast and non-invasive compared with other methods, and turtles can escape the stimuli immediately by moving their hind limb. Owing to these advantages, many nociceptive tests in turtles (13, 29, 30) have been carried out by this method. The TWL evaluated by Hargreaves's device has proven to be a sound and reproducible measure of complex nociceptive behaviour (3), and it has been extensively used for pain assessment in reptiles (6, 19, 28-30).

In an earlier pilot trial conducted on another group of turtles (to set up the experimental method), it was observed that the amount of water residue on the turtles' limbs could affect TWL measurement. This was in line with a previous study characterizing the variables in TWL measurement (3). To eliminate this factor, the turtles' hind limbs were completely and consistently dried just before blood collection, and after each animal had been examined, the box surface had to be dried. This procedure was essential to reduce variability in the study.

In the present study, a different temperature (70° rather than 50°C) was set for the beam source compared to previous studies (13, 29, 30). This change was needed because the PK/PD design was contingent on the blood collection and TWL measurement occurring together. If the 50°C setting had been used, the result could have taken several minutes, making the PK/PD protocol assumption invalid.

After the IM injection of TAP, plasma drug concentrations were detectable for up to 24 h. This persistence was longer than that reported in cats (8 h) and goats (6 h) despite the same dose and route being used (Prof. E. Lavy et al., unpublished data) (22). TAP in turtles showed slower absorption ($T_{max} = 1.42$ h) than in cats ($T_{max} = 0.25$ h) (22) and goats ($T_{max} = 0.17$ h; Lavy et al., unpublished data). Furthermore, TAP in turtles had a half-life almost double that reported in cats ($T_{1/2} = 4.04$ h vs 2.28 h) (22). TAP is metabolized predominantly by glucuronidation in humans (31), as 83% of an oral dose of TAP is converted into and excreted as an inactive glucuronated metabolite. Compared to mammals, turtles have a lower liver metabolic capacity and a slower metabolic rate (2, 24). These differences may have contributed to the long terminal half-life value of TAP found in turtles.

In a previous study, morphine (6.5 mg/kg SC) produced analgesic effect between 2 and 24 h in turtles (30). Tramadol (10 mg/kg SC) produced a long-lasting analgesic effect between 6 and 48 h (1). According to Sladky et al. (29), thermal antinociception of opioids in turtles appeared to be attributable mainly to MOR activation with a relatively minor contribution of delta-opioid receptor activation. It was assumed that the analgesic effect continued from 2 to 8 h after the administration of an experimental MOR agonist ((D-Ala², N-Me-Phe⁴, Gly⁵-ol)-enkephalin acetate salt) by SC at a dose of 6.6 mg/kg (29). When TAP (5 mg/kg IM) was administered to turtles, the analgesic effect occurred within 1 h and lasted for 10 h after administration. Compared with other studies, TAP produced a thermal antinociception effect more rapidly than morphine and tramadol in turtles, despite a lower dose. It is likely that the onset of the analgesic effect of TAP depends on the different administration route used (IM vs. SC). Classical MOR agonists (eg morphine) cause a long-lasting respiratory depression in turtles (30) because of their strong MOR activation. The present study did not evaluate the respiratory rate. It is expected, however, that TAP would cause less respiratory depression, because of its lower MOR affinity compared to morphine and M1 (34).

The TAP plasma concentration and effect vs. time curves have shown to be in phase (Fig. 3A). Indeed, when antinociception effect is plotted against plasma concentration, the plasma concentration and effect form a linear correlation ($r^2 = 0.98$) (Fig. 3B). This varies from an earlier study reporting PK/PD of buprenorphine in cats (27). The linear relationship between the TAP plasma concentration and effect might be accounted for by the rapid blood-brain equilibration and its high

MOR affinity (33). This is consistent with the high lipophilicity of TAP (5).

In humans, the Minimal Effective Concentration (MEC) of TAP is reported to be 0.67 $\mu\text{mol/L}$, equivalent to 148 ng/mL (33). The TAP plasma concentration in turtles exceeded this threshold from 1 to 10 h (Fig. 1), and during this time the thermal antinociceptive effect was noted. However, the extrapolation of the MEC value from humans to animals should be done with caution (18) and verified by larger sample size animal studies.

Compared to other animal species, turtles were slow in absorbing and eliminating TAP after IM injection. The thermal antinociceptive effect occurred within 1 h, reached its peak at 1 h, lasted for at least 10 h and was plasma-concentration dependent. TAP appears to be an attractive option for antinociception in turtles because of the rapid onset and acceptable duration of its effect. However, before adopting it for use in reptile clinical practice, it is necessary to consider systemic pharmacokinetics and biophase distribution characteristics, as well as to make a sound assessment of the drug's safety.

Conflict of interest statement. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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