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Pharmacokinetic and Pharmacodynamic Studies of Tapentadol and its Enhanced Antinociceptive Effect by Flupirtine

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General Introduction

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Bonica, 1979). However, this definition is described in subjective language and is difficult to ascribe to animals. There is no standard definition of pain in animals. According to Molony and Kent (1997), pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. Pain changes the animal's physiology and behavior to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery. Pain relies on the activation of a distinct mode of receptors.

Pain caused by various factors can be classified into three major types, including nociceptive, inflammatory, and neuropathic pain. Nociceptive pain, generally of acute origin, is caused by thermal, mechanical or chemical stimulations of peripheral nerve fibers. Inflammatory pain is the result of tissue injury and inflammation, and is represented in absence of any peripheral nerve damage. Neuropathic pain is caused by damage or disease affecting any part of the nervous system involved (Ochoa, 2009), and is characterized by remarkable plasticity that is the combination of sensory loss with paradoxical hypersensitivity (e.g. allodynia, hyperalgesia) (Woolf et al., 1999). These types exist in form of acute or chronic conditions. Acute and

chronic pains show different time courses. Acute pain does not outlast the healing process, whereas chronic pain lasts beyond the healing time for an injury. Persistent pain plays an important role in the conversion from acute to chronic pain conditions (Kehlet et al., 2006). Clinical classification of chronic pain is still controversial, although often classified by an associated disease (diabetic neuropathy, cancer pain, arthritis). It is characterized by extended duration and represents a complex of pathophysiologic actions (Fox, 2009), being frequently difficult to treat with a single analgesic and shows unpredictable response to analgesics (Martin and Eisenach, 2001).

From the animal welfare aspect, pain is still a huge concern; farm animals are routinely subjected to painful procedures with no analgesics; perioperative pain management in small and exotic animals is inconsistent; and management of cancer-related and chronic pain remains a challenge (Egger et al., 2013). Pain may adversely affect the animal's quality of life (QOL) due to the distress originating from inability to avoid damage. Pain exerts a harmful influence on conditions of animals such as appetite, behavior and intestinal function. Furthermore, systemic problems can be caused by neglect of pain (Egger et al., 2013).

The management of pain is an important consideration in human medicine. Likewise, pain management is regarded as an essential clinical component in modern veterinary medicine. As mentioned above, pain

management is central to animal welfare and it has been shown to affect animal production. For the treatment of pain, veterinary clinicians have a number of options consisting of several therapeutic categories such as anticonvulsants, α_2-agonists, local anesthetics, opioids and non-steroidal anti-inflammatory drugs (NSAID). Local anesthetics and NSAIDs are the most common drugs used clinically which act at the periphery (Viñuela-Fernández et al., 2007). Opioids and α_2-agonists are widely used to provide analgesic effects by acting on the central nervous system (Robertson and Taylor, 2004). Opioids have provided the most effective analgesia and are the main drug used in both acute and chronic pain management (Fox, 2014). Although NSAIDs are effective in pain control, NSAIDs are inadequate for the treatment of severe pain because of their lower efficacy compared with opioids. Although inflammatory pain responds well to NSAIDs, but neuropathic pain due to nerve damage or neural dysfunction dose not (Woolf et al., 1999).

Opioids are widely used to treat pain in both animals and humans and are considered to be the most effective and dependable drugs for controlling pain in mammals (Egger et al., 2013). Opioids consist of several therapeutic categories such as the classical μ-opioid receptor (MOR) agonists (e.g. morphine), the partial MOR agonists (e.g. buprenorphine), the mixed opioid κ-opioid receptor (KOR) agonists MOR-antagonists (e.g. butorphanol), and

the atypical opioids (e.g. tramadol). Although there are four currently recognized opioid receptors including MOR (μ), KOR (κ), DOR (δ) and nociception/orphanin FQ peptide receptor (NOR) (Egger et al., 2013). MOR and KOR are the main targets in veterinary medicine for pain control. MOR agonists produce more profound analgesic effect as well as more adverse effect than KOR agonists in many species (Wright, 2002). KOR agonists are also reported to have some central side effects such as dysphoria and sedation (Martin and Eisenach, 2001). For these reason, MOR agonists have been used for decades in the treatment of moderate to severe pain in both human and veterinary medicine (Meldrum, 2003). However, classic MOR agonists, such as morphine, are less effective against chronic pain of neuropathic or inflammatory origin, although they respond well to acute pain (Kalso et al., 2004). This decrease of effectiveness for chronic pain is caused by MOR down regulation with long-term therapies which requires increasing dose (Dickenson and Suzuki, 2005). Furthermore, classic MOR agonists are associated with serious side effects such as nausea, emesis, constipation and respiratory depression, limiting their usefulness for the treatment of chronic pain. As a consequence, there is still a need to find new agents having a better efficacy and safety profile for the treatment of chronic pain.

An alternative approach to improve the efficacy and safety profile is to combine the MOR activation with an additional mode of action (Tzschentke

et al., 2006a). Such an additional mechanism may diminish the otherwise side effects of the MOR activation (Schroder et al., 2011; Tzschentke et al., 2006a). Recently, an approach combing MOR activation with norepinephrine (NE) and/or serotonin (5-hydroxytrypta-mine [5-HT]) reuptake inhibition has been undertaken to improve the therapeutic range of opioid analgesics (Tzschentke et al., 2006a; 2007). Agents that block the reuptake of NE and/or 5-HT are effective analgesics in the treatment of chronic neuropathic pain conditions (Carter and Sullivan, 2002). Moreover, these agents potentiate the analgesic effects of opioids such as morphine (Ossipov et al., 1982). Overall, the analgesic mechanisms of MOR activation and NA/5-HT reuptake inhibition manifest complementary modes of action, and compounds with combined mechanisms of action may be better suited for the treatment of chronic pain (Kress, 2010; Tzschentke et al., 2006a).

Tramadol (TMD) is the first molecule with dual mechanisms of action and produces MOR activation and inhibition of 5-HT and NE reuptake. It was first developed in 1962 and has been used for the treatment of pain in Germany (Schenck and Arend, 1978). Moreover, it is marketed for pain management for cats and dogs in Italy. TMD is a racemate with two enantiomers, both of which contribute to analgesic effect via different mechanisms (Grond and Sablotzki, 2004). The MOR activation of TMD resides in the (+)-tramadol and the metabolite (+)-O-desmethyl-tramadol

(M1). On the other hand, the inhibitory activities of 5-HT and NE reuptake reside in (+)- and (-)-tramadol, respectively (Grond and Sablotzki, 2004). Moreover, most of its effect is the result of the active metabolite M1 (Tzschentke et al., 2007). This relative contribution of the mechanisms of action has been attributed to an unpredictable analgesic effect. Furthermore, the formation of M1 depends on the cytochrome P450 (CYP2D6), which is polymorphic in humans, and the administration of TMD with standard doses produces an unpredictable analgesic effect in humans (Poulsen et al., 1996). TMD is metabolized faster to inactive metabolites N-desmethyl-tramadol (M2) and O,N-didesmethyl-tramadol (M5), in many animal species (Black et al., 2010; Cox et al., 2011; Giorgi et al., 2009a; 2009b; 2009c; 2009d; 2009e; KuKanich and Papich, 2004; Souza and Cox, 2011). The clinical analgesic effect of TMD is uncertain for some animals, particularly in species that metabolize the molecule to inactive metabolites (Giorgi, 2008; Giorgi et al., 2009c). Recently, PK/PD studies of TMD in dogs have been demonstrated that it has not shown analgesic effects in the mechanical and thermal nociception test, due to the lack of M1 (Kogel et al., 2014; KuKanich and Papich, 2011). Therefore, it is possible that tramadol may not provide as effective and safe treatment for pain as in humans (De Sousa et al., 2008; Giorgi et al., 2009a; 2009b; 2009c; 2009d; 2009e; 2010). Although, tramadol has been reported to be effective in a small number of clinical investigations

(Pypendop et al., 2009; Vettorato et al., 2010), the real efficacy of tramadol in veterinary medicine is still controversial.

Tapentadol (TAP) is a novel centrally acting analgesic that combines two different mechanisms of action, MOR agonist and NE reuptake inhibition (Tzschentke et al., 2007). It has recently been added to the atypical opioid class. TAP was first developed in Germany and was launched on the European market for human use in 2011. In addition, the US FDA approved it in 2008 for the treatment of moderate to severe pain (Hartrick and Rozek, 2011). Chemically, TAP is 3-[(1R,2R)-3-(dimethylamino)-1-ethyl-2-methylpropyl] phenol hydrochloride with two chiral centers (Fig. 1A). It has a structure similar to tramadol (Fig.1B). TAP has four stereoisomers as follows SS, RS, SR and RR forms, only the RR form being approved as analgesic (Jain and Basniwal, 2013). The binding affinity to MOR is approximately 10 folds higher than to KOR and DOR (Tzschentke et al., 2006a; 2007). Although MOR affinity of TAP is approximately 50 folds lower than that of morphine, its analgesic potency in a variety of preclinical analgesia models was only 2-3 folds lower than that of morphine (Tzschentke et al., 2007). The effect of NE reuptake inhibition by TAP was similar to that of serotonin norepinephrine reuptake inhibitors (SNRI, e.g. venlafaxine) in the rat (Tzschentke et al., 2007). Despite the low MOR affinity of TAP, only a slight decrease of analgesic potency was observed,

which suggests that the NE reuptake inhibitory effect contributes to its analgesic effects (Tzschentke et al., 2006b; 2007). Moreover, a preclinical research in a neuropathic pain model showed that TAP acted primarily in the spinal cord and its analgesic effect is depended solely on MOR activation and NE reuptake inhibition (Bee et al., 2011). TAP also produced the antinociceptive effect in a rodent model for diabetic neuropathic pain (Christoph et al., 2010). Thus, TAP appears to have analgesic effects in both acute pain and in chronic neuropathic pain.

Furthermore, TAP is consistently two to five times more potent than TMD in both the tail flick test and the rat spinal nerve ligation model (Raffa et al., 2012). Analgesic effect of TMD is only arisen from its M1, showing many other futile metabolites and the other inefficacious (-)-enantiomer out of the racemic mixture. As opposed to the metabolic profile of TMD, TAP is metabolized predominantly by O-glucuronidation to O-glucuronide, which does not show any affinity for MOR and the NE transporter (Tzschentke et al., 2007). Thus, there are no active metabolites of TAP and metabolic activation is not required for analgesic effect, in contrast to TMD. Accordingly, TAP could potentially overcome a number of the disadvantages of TMD, since TAP is a pure enantiomer of RR and the parent compound is solely responsible for its pharmacological activity.

As mentioned above, a typical MOR agonist like morphine produces side effects such as nausea and emesis, constipation, respiratory depression, addiction, and dependence. According to Matthes et al., (1996), these side effects of opioids are mediated by the same MOR subtype. As a consequence, it is expected that TAP produces fewer opioid related side effects than classical MOR agonists. When compared to classical opioids, TAP induced much less nausea and vomiting in ferrets, and the duration of side effects was also shorter (Tzschentke et al., 2009). Moreover, the threshold dose for these effects was 100 times higher for TAP than for morphine. In accordance with these data, TAP had a favorable tolerability than morphine at equianalgesic intraperitoneal doses in humans on both (i) gastrointestinal motility assessed from charcoal transit and (ii) prostaglandin-induced diarrhea. Additionally, systemic administration of TAP in humans is associated with a 2-3 folds reduction in the rate of adverse effects reported with oxycodone (Biondi et al., 2013) and a better tolerance and physical dependence profile was observed (Tzschentke et al., 2006b; 2007). Overall, TAP could be a promising novel analgesic with low side effects, however much more data is required before it can be recommended for regular use in veterinary medicine.

Veterinary medicine faces the unique challenge of having to treat many animal species, including mammals, birds, reptiles and fishes. The main

challenge for veterinarians is not just to select a drug but to determine, for the selected agent, a rational dosing regimen which is a long and complicated endeavor because of differences in the expression of enzymes, receptors and signal transduction molecules between species (Giorgi, 2012). Both inter- and intra-species differences in drug response can be attributed to either variations in pharmacokinetics (PK) or pharmacodynamics (PD), the magnitude of which varies from drug to drug (Riviere et al., 1997). Generally, the action of each drug depends on the concentration–time profile at the site of action; this concept provides a basis for improved drug development through the use of PK/PD modeling (Toutain and Lees, 2004). The PK and PD data generated in the single dose study is bridged using a link model and this linked information may be used as an alternative and preferred tool to select rational dosage regimens (both dose and dosing interval) for further evaluation in clinical trials. PK/PD assessment is the major tool for rational dosage regimen determination, as it quantifies the two main sources of interspecies variability. Hence, PK/PD studies are critical when a drug is applied to a new animal species.

The situation of treatment with two or more drugs is common in clinical care of humans and animals. The use of multiple drugs that have different mechanisms of action may produce the effect more efficaciously against a single target or a disease. As described before, the use of classic

MOR agonists seem to be remarkably effective against acute pain, but less suited to chronic neuropathic pain. Moreover, MOR agonists are accompanied by severe side effects. In certain cases, drug combination of opioids and analgesics that have different mechanisms of action, may result in a synergistic analgesic effect, dose and toxicity reduction, and minimize or delay the induction of drug resistance (Chou, 2006). This multimodal therapy may offer enhanced effects compared to equianalgesic doses of the individual drugs in complex pain condition. Thus, the ideal combination regimen would both enhance analgesic efficacy and reduce side effects compared to a single drug.

Several combination studies with classic opioids (particularly morphine) have reported that multimodal therapies enhanced analgesic effects in various animal models of nociception (Argüelles et al., 2002; Hernández-Delgadillo et al., 2002; Kolosov et al., 2012; Miranda et al., 2013; Ossipov et al., 1997). Specifically, combination investigations of opioids and NSAIDs have been investigated in different animal models: tramadol with meloxicam in the sciatic nerve ligated rat model as well as in the formalin test, tramadol with metamizol in hind paw test, buprenorphine with lumiracoxib in the rat orofacial formalin test (Abass et al., 2014; Capuano et al., 2009; Hernández-Delgadillo et al., 2002; Isiordia-Espinoza et al., 2011). Recently, a combination investigation of TAP and pregablin has reported

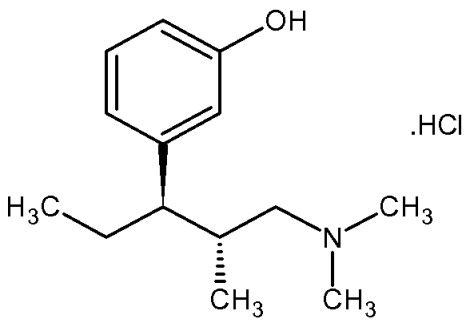
that the combination resulted in a synergistic antihypersensitive activity in a rat model of neuropathic pain (Christoph et al., 2011). Accordingly, it could be expected that the combination of TAP with analgesics with different mechanisms of action would improve the efficacy and tolerability profiles in animals.

Flupirtine (FLP) is a centrally acting, non-opioid analgesic that belongs to the triaminopyridine class. It was approved in the 1980s in Germany for the treatment of several pain states (Devulder, 2010). FLP acts as a selective neuronal potassium channel opener having N-methyl-D-aspartate (NMDA) receptor antagonism (Kornhuber et al., 1999), however, the exact mechanism of action has remained unknown until recently. In addition, it has muscle relaxant and anticonvulsant effect in pain management. These properties contribute to its therapeutic advantages without the side effects of classic opioids or NSAIDs (Szelenyi and Nickel, 1991). In earlier studies, FLP has been reported to have no interaction with serotonin, dopamine, nicotine receptors or adrenoceptors (Bleyer et al., 1988). In spite of these benefits, the use of FLP has been limited due to the side effects such as somnolence and dizziness. Consequently, its use has been limited to mild or moderate musculoskeletal pain syndromes in humans. However, a molecule with NMDA antagonism is likely to have synergistic or additive interactions with other analgesics such as morphine (Goodchild et al., 2008a). Several

investigations have been reported that FLP enhanced analgesic effects of opioids in various animal pain models (Capuano et al., 2011; Goodchild et al., 2007; 2008a; 2008b; Kolosov et al., 2012). Previous studies suggest that the combination of TAP and FLP may display the synergistic interaction.

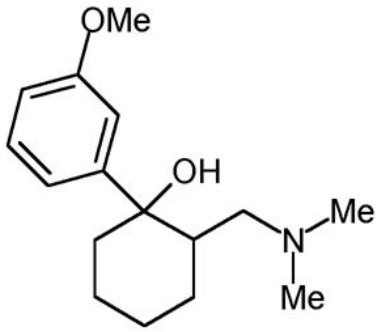
TAP is known to yield high and reliable analgesic effect for the treatment of chronic pain with low side effects in humans, as compared to classic opioids. If the pronounced efficacy and safety profiles of TAP holds true in animals, it would be a useful drug for the treatment of chronic pain in companion, industrial and even exotic animals. However, much more data in variety of animal species is needed to propose its use in veterinary medicine. The aim of this study are: i) to assess the pharmacokinetics of TAP after IV, IM and SC injection in healthy cats; ii) to assess the pharmacokinetics of TAP after IV and IM injection in healthy goats; iii) to evaluate the PK/PD relationship in turtles, after a single IM injection of TAP; iv) to determine the antinociceptive effect of TAP and FLP in rats when administered separately or in combination, as well as their synergistic interaction.

A)



3-[(1R,2R)-3 (dimethylamino)-1-ethyl-2- methylpropyl] phenol hydrochloride

B)



(1RS,2RS)- 2-[(dimethylamino)methyl]- 1 -(3-methoxyphenyl)- cyclohexanol

Fig. 1. Chemical structure of tapentadol hydrochloride (A) and tramadol (B)

References

1. Bee, L.A., Bannister, K., Rahman, W., Dickenson, A.H., 2011. Mu-opioid and noradrenergic α_2-adrenoceptor contributions to the effects of tapentadol on spinal electrophysiological measures of nociception in nerve-injured rats. *Pain* 152, 131–139.

2. Biondi, D., Xiang, J., Benson, C., Etropolski, M., Moskovitz, B., Rauschkolb, C., 2013. Tapentadol immediate release versus oxycodone immediate release for treatment of acute low back pain. *Pain Physician* 16, E237–246.

3. Black, P.A., Cox, S.K., Macek, M., 2010. Pharmacokinetics of tramadol hydrochloride and its metabolite O-desmethyltramadol in peafowl (*Pavo cristatus*). *Journal of Zoo and Wildlife Medicine* 41, 671–676.

4. Bleyer, H., Bleyer, H., Carlsson, K.-H., Carlsson, K.H., Erkel, H.J., Erkel, H.-J., Jurna, I., Jurna, I., 1988. Flupirtine depresses nociceptive activity evoked in rat thalamus. *European journal of Pharmacology* 151, 259–265.

5. Bonica, J.J., 1979. The need of a taxonomy. *Pain* 6, 247–248.

6. Capuano, A., De Corato, A., Treglia, M., Tringali, G., Navarra, P., 2011. Flupirtine antinociception in the rat orofacial formalin test: an analysis of combination therapies with morphine and tramadol. *Pharmacology, Biochemistry and Behavior* 97, 544–550.

7. Carter, G.T., Sullivan, M.D., 2002. Antidepressants in pain management. *Current Opinion in Investigational Drugs* 3, 454–458.

8. Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews* 58, 621–681.

9. Christoph, T., De Vry, J., Tzschentke, T.M., 2010. Tapentadol, but not morphine, selectively inhibits disease-related thermal hyperalgesia in a mouse model of diabetic neuropathic pain. *Neuroscience Letters* 470, 91–94.

10. Cox, S., Martin Jimenez, T., Van Amstel, S., Doherty, T., 2011. Pharmacokinetics of intravenous and intramuscular tramadol in llamas. *Journal of Veterinary Pharmacology and Therapeutics* 34, 259–264.

11. De Sousa, A.B., Santos, A.C.D., Schramm, S.G., Porta, V., Górniak, S.L., Florio, J.C., De Souza Spinosa, H., 2008. Pharmacokinetics of tramadol and o-desmethyltramadol in goats after intravenous and oral administration. *Journal of Veterinary Pharmacology and Therapeutics* 31, 45–51.

12. Devulder, P.D.J., 2010. Flupirtine in Pain Management. *CNS Drugs* 24, 867–881.

13. Dickenson, A.H., Suzuki, R., 2005. Opioids in neuropathic pain: clues from animal studies. *European Journal of Pain* 9, 113–116.

14. Egger, C.M., Love, L., Doherty, T., 2013. Pain Management in Veterinary Practice, 1st ed. John Wiley & Sons.

15. Fox, S.M., 2014. Pain Management in Small Animal Medicine, 1st ed. CRC Press.

16. Fox, S.M., 2009. Chronic Pain in Small Animal Medicine, 1st ed. CRC Press.

17. Giorgi, M., 2008. Pharmacokinetic differences of tramadol in several animal species and human beings. *Journal of Veterinary Resarch* 63, 1–4.

18. Giorgi, M., 2012. Veterinary pharmacology: is it still pharmacology's cinderella? *Clinical and Experimental Pharmacology* 2, 2-2.

19. Giorgi, M., Del Carlo, S., Łebkowska-Wieruszewska, B., Kowalski, C., Saccomanni, G., 2010. Pharmacokinetics of tramadol and metabolites after injective administrations in dogs. *Polish Journal of Veterinary Sciences* 13, 639–644.

20. Giorgi, M., Del Carlo, S., Saccomanni, G., Łebkowska-Wieruszewska, B., Kowalski, C.J., 2009a. Pharmacokinetics of tramadol and its major metabolites following rectal and intravenous administration in dogs. *New Zealand Veterinary Journal* 57, 146–152.

21. Giorgi, M., Del Carlo, S., Saccomanni, G., Łebkowska-Wieruszewska, B., Kowalski, C.J., 2009b. Pharmacokinetic and urine profile of tramadol and its major metabolites following oral immediate release capsules administration in dogs. *Veterinary Research Communications* 33, 875–885.

22. Giorgi, M., Del Carlo, S., Saccomanni, G., Łebkowska-Wieruszewska, B., Turini, V., Kowalski, C., 2009c. Biopharmaceutical profile of tramadol in the dog. *Veterinary Research Communications* 33, 189–192.

23. Giorgi, M., Del Carlo, S., Sgorbini, M., 2009d. Pharmacokinetics of tramadol and its metabolites M1, M2, and M5 in donkeys after

intravenous and oral immediate release single-dose administration. *Journal of Equine Veterinary Science* 29, 569–574.

24. Giorgi, M., Saccomanni, G., Łebkowska-Wieruszewska, B., Kowalski, C., 2009e. Pharmacokinetic evaluation of tramadol and its major metabolites after single oral sustained tablet administration in the dog: a pilot study. *The Veterinary Journal* 180, 253–255.

25. Goodchild, C., Kolosov, A., Tucker, A., Nadeson, R., 2007. Synergistic interactions between a KCNQ channel opener and an opioid: flupirtine and morphine in rat pain models including neuropathic pain. *Pain* 6, 611-618.

26. Goodchild, C.S., Kolosov, A., Tucker, A.P., Cooke, I., 2008a. Combination therapy with flupirtine and opioid: studies in rat pain models. *Pain Medicine* 9, 928–938.

27. Goodchild, C.S., Nelson, J., Cooke, I., Ashby, M., Jackson, K., 2008b. Combination therapy with flupirtine and opioid: open-label case series in the treatment of neuropathic pain associated with cancer. *Pain Medicine* 9, 939–949.

28. Grond, D.S., Sablotzki, A., 2004. Clinical pharmacology of tramadol. *Clinical Pharmacokinetics* 43, 879–923.

29. Hartrick, D.C.T., Rozek, R.J., 2011. Tapentadol in pain management. *CNS Drugs* 25, 359–370.

30. Jain, D., Basniwal, P.K., 2013. Tapentadol, a novel analgesic: Review of recent trends in synthesis, related substances, analytical methods,

pharmacodynamics and pharmacokinetics. *Bulletin of Faculty of Pharmacy, Cairo University* 51, 283–289.

31. Kalso, E., Edwards, J.E., Moore, R.A., McQuay, H.J., 2004. Opioids in chronic non-cancer pain: systematic review of efficacy and safety. *Pain* 112, 372–380.

32. Kehlet, H., Jensen, T.S., Woolf, C.J., 2006. Persistent postsurgical pain: risk factors and prevention. *The Lancet* 367, 1618–1625.

33. Kogel, B., Terlinden, R., Schneider, J., 2014. Characterisation of tramadol, morphine and tapentadol in an acute pain model in Beagle dogs. *Veterinary Anaesthesia and Analgesia* 41, 297–304.

34. Kolosov, A., Goodchild, C.S., Williams, E.D., Cooke, I., 2012. Flupirtine enhances the anti-hyperalgesic effects of morphine in a rat model of prostate bone metastasis. *Pain Medicine* 13, 1444–1456.

35. Kornhuber, J., Bleich, S., Wiltfang, J., Maler, M., Parsons, C.G., 1999. Flupirtine shows functional NMDA receptor antagonism by enhancing Mg^{2+} block via activation of voltage independent potassium channels. *Journal of Neural Transmission* 106, 857–867.

36. Kress, H.G., 2010. Tapentadol and its two mechanisms of action: is there a new pharmacological class of centrally-acting analgesics on the horizon? *European Journal of Pain* 14, 781–783.

37. KuKanich, B., Papich, M.G., 2004. Pharmacokinetics of tramadol and the metabolite O-desmethyltramadol in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 27, 239–246.

38. KuKanich, B., Papich, M.G., 2011. Pharmacokinetics and antinociceptive effects of oral tramadol hydrochloride administration in Greyhounds. *American Journal of Veterinary Research* 72, 256-262.

39. Martin, T.J., Eisenach, J.C., 2001. Pharmacology of opioid and nonopioid analgesics in chronic pain states. *The Journal of Pharmacology and Experimental Therapeutics* 299, 811–817.

40. Matthes, H.W.D., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dollé, P., Tzavara, E., Hanoune, J., Roques, B.P., Kieffer, B.L., 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature* 383, 819–823.

41. Meldrum, M., 2003. Opioids and pain relief. IASP Press.

42. Molony, V., Kent, J.E., 1997. Assessment of acute pain in farm animals using behavioral and physiological measurements. *Journal of Animal Science* 75, 266–272.

43. Ochoa, J.L., 2009. Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology* 72, 1282–1283.

44. Ossipov, M.H., Malseed, R.T., Goldstein, F.J., 1982. Augmentation of central and peripheral morphine analgesia by desipramine. *Archives Internationales de Pharmacodynamie et de Therapie* 259, 222–229.

45. Poulsen, L., Arendt-Nielsen, L., Brøsen, K., Sindrup, S.H., 1996. The hypoalgesic effect of tramadol in relation to CYP2D6. *Clinical Pharmacology and Therapeutics* 60, 636–644.

46. Pypendop, B.H., Siao, K.T., Ilkiw, J.E., 2009. Effects of tramadol hydrochloride on the thermal threshold in cats. *American Journal of Veterinary Research* 70, 1465–1470.

47. Raffa, R.B., Buschmann, H., Christoph, T., Eichenbaum, G., Englberger, W., Flores, C.M., Hertrampf, T., Kogel, B., Schiene, K., Straßburger, W., Terlinden, R., Tzschentke, T.M., 2012. Mechanistic and functional differentiation of tapentadol and tramadol. *Expert Opinion on Pharmacotherapy* 13, 1437–1449.

48. Riviere, J.E., Martin Jimenez, T., Sundlof, S.F., Craigmill, A.L., 1997. Interspecies allometric analysis of the comparative pharmacokinetics of 44 drugs across veterinary and laboratory animal species. *Journal of Veterinary Pharmacology and Therapeutics* 20, 453–463.

49. Robertson, S.A., Taylor, P.M., 2004. Pain management in cats--past, present and future. Part 2. Treatment of pain-clinical pharmacology. *Journal of Feline Medicine and Surgery* 6, 321–333.

50. Schenck, E.G., Arend, I., 1978. The effect of tramadol in an open clinical trial. *Arzneimittel-Forschung* 28, 209–212.

51. Schroder, W., Tzschentke, T.M., Terlinden, R., De Vry, J., Jahnel, U., Christoph, T., Tallarida, R.J., 2011. Synergistic interaction between the two mechanisms of action of tapentadol in analgesia. *Journal of Pharmacology and Experimental Therapeutics* 337, 312–320.

52. Souza, M.J., Cox, S.K., 2011. Tramadol use in zoologic medicine. *The Veterinary Clinics of North America, Exotic animal practice* 14, 117–130.

53. Szelenyi, I., Nickel, B., 1991. Pharmacological profile of flupirtine, a novel centrally acting, non-opioid analgesic drug. *Agents Actions Suppl* 32, 119–123.

54. Toutain, P.L., Lees, P., 2004. Integration and modelling of pharmacokinetic and pharmacodynamic data to optimize dosage regimens in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics* 27, 467–477.

55. Tzschentke, T.M., Christoph, T., Kogel, B., Schiene, K., Schiene, K., Hennies, H.H., Englberger, W., Haurand, M., Jahnel, U., Cremers, T.I.F.H., Friderichs, E., De Vry, J., 2007. (-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride (tapentadol HCl): a novel mu-opioid receptor agonist/norepinephrine reuptake inhibitor with broad-spectrum analgesic properties. *The Journal of Pharmacology and Experimental Therapeutics* 323, 265–276.

56. Tzschentke, T.M., De Vry, J., Terlinden, R., Hennies, H.H., Lange, C., Strassburger, W., Haurand, M., Kolb, J., Schneider, J., Buschmann, H., Finkam, M., Jahnel, U., Friderichs, E., 2006. Tapentadol hydrochloride. *Drugs of the Future* 31, 1053–1061.

57. Tzschentke, T.M., Jahnel, U., Kogel, B., Christoph, T., Englberger, W., De Vry, J., Schiene, K., Okamoto, A., Upmalis, D., Weber, H., Lange, C., Stegmann, J.-U., Kleinert, R., 2009. Tapentadol hydrochloride: a next-generation, centrally acting analgesic with two mechanisms of action in a single molecule. *Drugs Today* 45, 483–496.

58. Vettorato, E., Zonca, A., Isola, M., Villa, R., Gallo, M., Ravasio, G., Beccaglia, M., Montesissa, C., Cagnardi, P., 2010. Pharmacokinetics and

efficacy of intravenous and extradural tramadol in dogs. *Veterinary Journal* 183, 310–315.

59. Viñuela-Fernández, I., Jones, E., Welsh, E.M., Fleetwood-Walker, S.M., 2007. Pain mechanisms and their implication for the management of pain in farm and companion animals. *Veterinary Journal* 174, 227–239.

60. Woolf, C.J., Woolf, C.J., Mannion, R.J., Mannion, R.J., 1999. Neuropathic pain: a etiology, symptoms, mechanisms, and management. *The Lancet* 353, 1959–1964.

61. Wright, B.D., 2002. Clinical pain management techniques for cats. *Clinical Techniques in Small Animal Practice* 17, 151–157.

Chapter 1. Pharmacokinetics of Tapentadol (TAP) after Intravenous (IV), Intramuscular (IM) and Subcutaneous (SC) Administration in Cats

Abstract

The aim of the present study was to investigate the pharmacokinetics of the novel atypical drug tapentadol (TAP) after intravenous (IV), intramuscular (IM) and subcutaneous (SC) injection in six healthy cats. The dose rate used was 5 mg/kg and the concentrations of TAP in plasma were evaluated using high-performance liquid chromatography.

Some adverse effects including salivation, agitation and panting, were noted, especially following IV administration. In all three administration groups, TAP concentrations were detectable in plasma for up to 8 h. Bioavailability for each route was almost complete, accounting for 94% and 90% after IM and SC administrations, respectively. Drug absorption was faster after IM than SC administration (0.25 h vs. 0.63 h). The half-life of the terminal portion of the plasma concentration curve was not significantly different between the three routes of administrations (2–3 h). TAP appears to have some variation in its pharmacokinetic features in cats compared to other animal species.

I. Introduction

As drug options to provide analgesia in cats are limited compared to those available for dogs, cats often receive inadequate analgesia, mainly because of the perceived risk of side effects and limited information on suitable alternatives (Lascelles et al., 1999). The investigation of new active ingredients suitable for feline therapy is therefore critical. Opioids are considered prototypical analgesics (Fox, 2010) and are used in veterinary medicine not only for analgesia but for their other clinical actions (e.g. anti-tussive, antidiarrheal and emetic). The classical strong MOR agonists can have significant adverse effects (Vadivelu et al., 2011) and are therefore generally licensed as controlled substances (Pascoe, 2000; Clutton, 2010). Therfore, atypical opioid drugs (especially tramadol) have gained popularity in small animal clinical practice.

Tramadol is one of the most widely sold atypical opioids. It is marketed for pain control of cats and dogs in Italy, although its real efficacy in dogs has been questioned (Giorgi, 2008; Giorgi et al., 2009). Since most of its effect is the result of the active metabolite M1, tramadol may not be safe for use in cats with liver disease. Tramadol possesses a weak agonist affinity for the MOR, reducing the typical opioid side effects, which are due to the activation of this receptor. However, its efficacy for pain relief, especially the relief of chronic pain, is enhanced by a second synergistic

mechanism of action, namely norepinephrine (NA) and serotonin (5-HT) reuptake (Raffa et al., 1992). Its application is generally limited to the treatment of mild to moderate pain and its effect is inferior to the strong classical opioids (morphine).

A new drug, tapentadol (TAP), has recently been added to the atypical opioid class. It was launched on the European market for human use in 2011. In humans, TAP has a lower incidence of adverse effects compared to equianalgesic doses of morphine (Kleinert et al., 2008) and oxycodone (Etropolski et al., 2011). TAP has attracted the attention of the veterinary world because its MOR affinity is 50-fold less than morphine but 120-fold higher than tramadol (Giorgi, 2012). Additionally, its second synergistic mechanism of action is known not to involve 5-HT reuptake, reducing the possibility of the 'serotonin storm effect' reported following rapid IV tramadol injections. In brief: (1) TAP is recommended in cases of moderate to severe pain (as is morphine); (2) compared to morphine, TAP produced much less nausea and vomiting and when these adverse effects were present, their duration was shorter (Tzschentke et al., 2009); (3) TAP is not restricted/regulated in most European countries; and (4) TAP does not require metabolic activation to be effective, so individual variations in drug metabolism should have limited effects on efficacy.

Very few studies to investigate the clinical uses of TAP have been undertaken in the veterinary field. The pharmacokinetic features of TAP have been investigated in dogs after IV and oral administration, demonstrating very low oral bioavailability (4%; Giorgi et al., 2012a). In a study of rabbits undergoing castration, it was reported that TAP had excellent efficacy for the reduction of surgical and post-surgical pain (Giorgi et al., 2013).

The aim of this study was to assess the pharmacokinetics of TAP after IV, IM and SC injection in healthy cats.

II. Materials and Methods

1. Drugs and reagents

TAP hydrochloride was supplied as a pure powder (> 99.8% purity; Bepharm, China). M1 was used as an internal standard and supplied as pure powder (> 99.8% purity; LCG Promochem, Germany). Additionally, high-performance liquid chromatography (HPLC) grade acetonitrile (ACN), dichloromethane (CH_2Cl_2) and diethyl ether (Et_2O) were used in the assays (Scharlau, USA), as was analytical grade acetic acid and sodium tetraborate decahydrate (BDH, Ireland). HPLC grade water was obtained by distilling deionised water produced by a Milli-Q Millipore water system (EDM Millipore, Italy). All the other reagents and materials were of analytical grade and supplied from commercial sources. The injectable solutions were prepared by dissolving the pure TAP hydrochloride powder in sterile saline to produce a 5 mg/mL solution, which was then passed through a 0.45 μm filter, maintaining sterile conditions.

2. Animals

Four male and two female mixed-breed cats, aged 3–6 years, with a bodyweight of 3.4–4.8 kg, were enrolled in the study. The cats were previously determined to be clinically healthy on physical examination,

serum chemistry and haematological analyses. Animal care and handling was 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 Lublin, which approved the study protocol.

3. Experimental design

Cats were randomly assigned to three treatment groups, using six slips of paper marked with the numbers 1–6, selected blindly from a box. An open, single-dose, three-treatment, three-period crossover design (3 x 3 Latin square) was used. All cats were fasted for 12 h overnight before each experiment. In the first period, each cat in group I (n = 2) received a single IV dose of TAP (5 mg/mL) at 5 mg/kg injected slowly over 2 minutes into the left jugular vein. This dose was selected based on previous information describing the effectiveness of TAP in laboratory species (Giorgi et al., 2013). Group II cats (n = 2) received a single IM injection of 5 mg/kg of TAP given into the rectus femoris portion of the quadriceps femoris muscle.

Wash out period is a 1-week for the complete metabolism and excretion of TAP. After this period, the groups were rotated and the experiment was repeated (second period). After a further interval of 1 week, the groups were rotated and the experiment was repeated (third period). By

the end of the study, each cat had received TAP by all the three administration routes.

A catheter was placed into the right cephalic vein to facilitate blood sampling. Blood samples (1 mL) were collected at 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 10 h after the administration of TAP and placed in collection tubes containing lithium heparin. All blood samples were centrifuged within 30 min of collection, at 3,000×*g*, 4°C for 15 min. The harvested plasma was stored at -70 °C and used within 15 days of collection.

4. High performance liquid chromatography (HPLC)

The concentration of TAP in plasma were determined using HPLC, according to the method previously described by Giorgi et al. (2012b). The analytical method was briefly re-validated in plasma from the cats. The HPLC system was an LC Waters (Waters, USA) consisting of quaternary gradient system (600 Controller, Waters, USA), in-line degasser (Waters, USA), photodiode array detector (2998 model, Waters, USA), multi lambda fluorescence detector (2475 model, Waters, USA) and auto sampler (model 717 plus, Waters, USA). Data was processed using Empower ProTM software (Waters, USA). The chromatographic separation assay was performed with a SunFire C18 analytical column (150 x 4.6 mm inner diameter, 5 μm particle size, Waters, USA) 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).

5. Preparation of plasma samples

Briefly, 50 μL of IS solution (0.5 μg/mL) and 0.2 mL 0.2 M 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 (Et_2O:CH_2Cl_2 7:3 v/v) was added, the tube was then vortexed (30 sec) and 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 0.5 mL polypropylene snap cap conical tube, placed in a vortex and then shaken with 0.2 mL of back-extraction solvent (0.05M HCl:ACN 1:1 v/v) for 5min, before being centrifuged for 10min at 15,625×*g*. The aqueous phase (50 μL) was injected into the HPLC system.

6. Pharmacokinetic evaluation

The pharmacokinetic calculations were carried out using WinNonlin v 5.3 (Pharsight). Maximum concentration (C_{max}) of TAP in plasma and the time required to reach C_{max} (T_{max}) were predicted from the data. The concentration at time 0 (C_0) for IV administration was estimated by back-extrapolating from the first two concentration values. 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 ($T_{1/2}\lambda z$) was calculated using $T_{1/2} = 0.693/\lambda$. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. The IM and SC bioavailabilities (F%) were calculated from the ratio of the areas under the plasma TAP concentration curve after IM or SC and IV administration, respectively, indexed to their respective dose:

$$F\ (\%) = (AUC_{IM/SC} \times Dose_{IV})/(AUC_{IV} \times Dose_{IM/SC}) \times 100$$

Changes in plasma concentration of TAP were evaluated using the standard non-compartmental analysis, and the relative pharmacokinetic parameters were determined using standard non-compartmental equations (Gabrielsson and Weiner, 2002.). Different models were assessed by visual inspection of the curve fits and the residuals' scatter plots, together with the

goodness of fit measures incorporated in the software (including the Akaike and Schwartz criteria).

7. Simulation of tapentadol (TAP) dosage regimens

A compartment open pharmacokinetic model was used to simulate the concentration–time profile for several dosage regimens after IM administration. Based on the pharmacokinetic analysis of pooled data, computer simulations (WinNonlin 5.3) were performed to calculate intramuscular dosage regimens that maintain TAP plasma concentrations greater than the minimal effective concentration (MEC) in human (148 ng/mL) for roughly 50% of the dose interval.

8. Statistical analysis

Pharmacokinetic data were evaluated using ANOVA tests to determine statistically significant differences. The pharmacokinetic parameters are presented as means ± standard deviation (SD) and the TAP plasma concentrations of each cat are presented as means. All analyses were conducted using GraphPad InStat (GraphPad Software). In all experiments, differences were considered significant if $P < 0.05$.

III. Results

After IV administration, some adverse effects including salivation, agitation and panting, were noted in all cats. However, they resolved rapidly and spontaneously within 20 min. These adverse effects were also detected after IM and SC administration, but were less intense and of a shorter duration. It was occurred in four cats (3/6 IM; 1/6 SC).

1. Validation of bioanalytical method

The HPLC method used was re-validated in feline plasma. Briefly, TAP was linear (r^2 > 0.98) in the range 10–4000 ng/mL. The intra-day repeatability was measured using coefficients of variation and was < 7.3%. Accuracy was measured by measuring proximity to the concentration added on the same replicates and was < 5.3%.

2. Pharmacokinetics of tapentadol (TAP)

In all three-administration groups, TAP concentrations were detectable in the plasma for up to 8 h. Some variability in plasma drug concentrations was detected among the cats and groups. Fig. 1 and 2 show individual (A–F) and average TAP plasma concentrations vs. time curves after each administration route, respectively. After IM injection, TAP showed variable

but fast absorption (T_{max} = 0.25 h, range 0.08–0.75 h), while after SC administration, absorption was significantly slower (T_{max} = 0.63 h). The $T_{1/2}$ λz was quite similar between the three administration routes in the range of about 2–3 h. Also Vz/F and Cl/F values were constant among the treatment groups. In the elimination phase of the curve, the decline of TAP was linear without any evidence of a secondary peak. The average pharmacokinetic parameters calculated for the three administrations are reported in Table 1. The bioavailabilities were almost complete, accounting for 94% and 90% after IM and SC administrations, respectively.

3. Simulation of tapentadol (TAP) dosage regimen

After pharmacokinetic simulation of IM multiple dosing, it was found that the plasma concentration when the TAP is administered at 5mg/kg q 24h is insufficient to exceed the MEC for 12 h (50% of dose interval). Following simulations of IM administration of TAP at doses of 3 mg/kg TID and 5 mg/kg BID, plasma concentrations were greater than the MEC value of 148 ng/mL for over 4.5 and 6 h, respectively (Fig. 3).

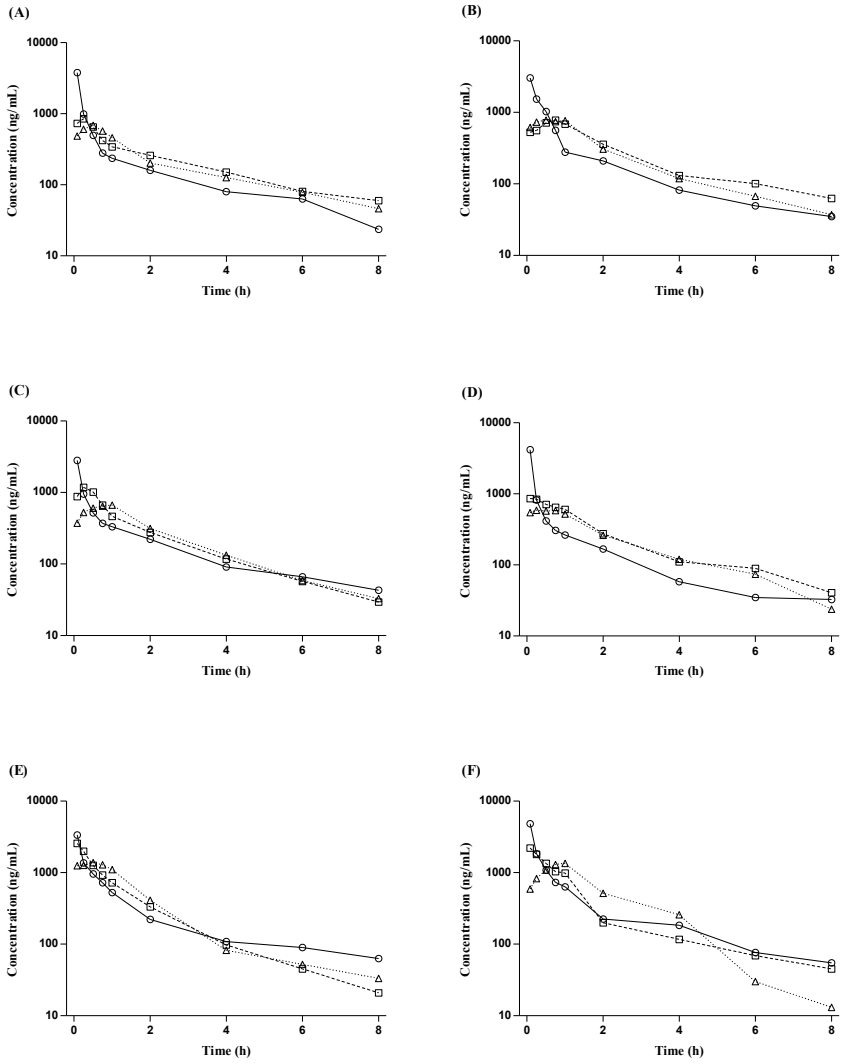


Fig. 1. Individual plasma concentration vs. time semilogarithmic curves (A–F) of tapentadol (TAP) after a single IV (circles), IM (squares) or SC (triangles) administration (n = 6).

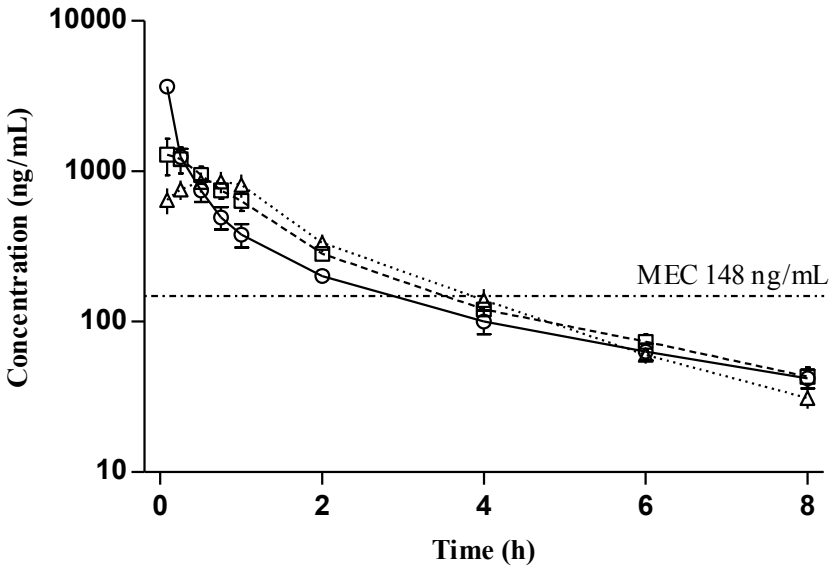


Fig. 2. Mean plasma concentration vs. time semilogarithmic curves of tapentadol (TAP) after a single IV (circles), IM (squares) or SC (triangles) administration (n = 6). The horizontal dotted line shows the minimal effective concentration (MEC, 148 ng/mL) reported for humans and its intercepts with the concentration vs. time curves reported in this feline study.

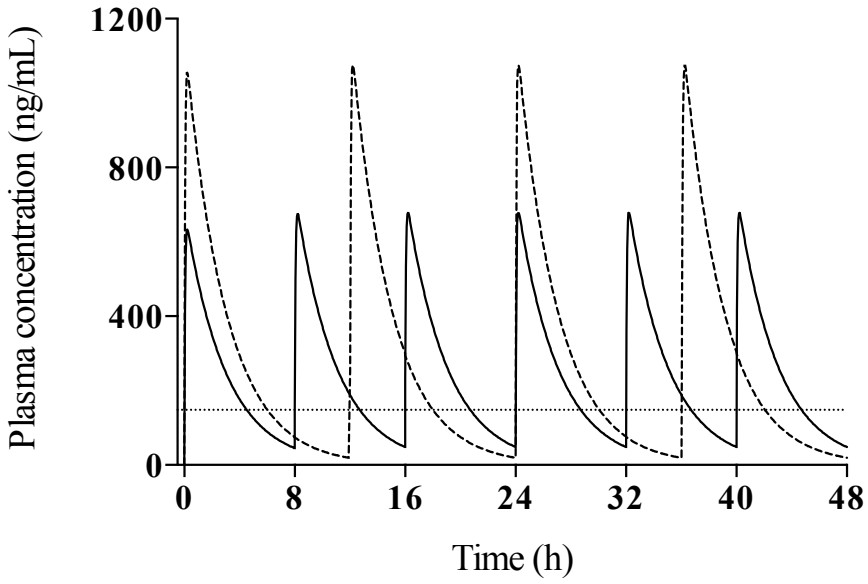


Fig. 3. Mean plasma concentrations vs. time curves of tapentadol (TAP) following a simulated IM multiple dose rate at 5 mg/kg BID (dotted line) and a simulated PO multiple dose rate at 3 mg/kg TID (solid line). The dashed line represents the minimal effective concentration (MEC; 148 ng/mL) in humans.

Table 1. Pharmacokinetic parameters after injection of tapentadol (TAP) at 5 mg/kg by IV, IM and SC route, respectively in cats (n = 6)

Phamacokinetic parameters (Unit)	IV (Mean±SD)	IM (Mean± SD)	SC (Mean± SD)
λz (1/h)	0.25±0.07	0.34±0.11	0.37±0.12
$T_{1/2}$λz (h)	2.93±0.86	2.28±0.85	2.05±0.6
T_{max} (h)	-	0.25±0.26 [a]	0.63±0.31 [a, b]
C_{max} (ng/mL)	-	1406±779 [a]	906±356 [a, b]
C_0 (ng/mL)	6289±1906	-	-
$AUC_{0-\infty}$ (h ng/mL)	2423±533	2245±343	2202±611
Vz/F (mL/kg)	8.79±1.97	7.53±2.95	7.06±2.10
Cl/F (mL/min/kg)	35.60±7.05	37.85±5.68	40.13±9.97
MRT (h)	2.44±0.87	2.55±0.94	2.35±0.34
F (%)	-	93.93±9.91	90.01±6.52 [a]

λz, first-order rate terminal elimination constant; $T_{1/2}$λz, half-life of the terminal portion of the curve; T_{max}, time at the maximum drug concentration; C_{max}, maximum drug plasma concentration; C_0, concentration at time 0; $AUC_{0-\infty}$, area under the curve from 0 to infinity; Vz/F, volume of distribution during the elimination phase; Cl/F, body clearance during the elimination phase; MRT, mean residence time; F%, bioavailability.

[a], $P < 0.05$ versus IV administration group.

[b], $P < 0.05$ versus IM administration group.

IV. Discussion

The side effects of TAP demonstrated in this study were very similar to those previously reported in dogs. However, cats became agitated, while dogs demonstrated depression (Giorgi et al., 2012a). This concurs with the contrasting behavior reported in cats and dogs after morphine administration (KuKanich and Papich, 2009). The side effects were more severe and of longer duration following IV than IM and SC administrations. This is most likely to be the result of high plasma drug concentrations achieved by the IV route of administration. None of the cats in this study vomited, which is a well-known side effect of morphine in cats (Taylor et al., 2001). Although an injectable pharmaceutical form of TAP is not currently available for purchase, the dose administered in the present study appears high when compared to the dose used in human clinical therapy (200 mg/patient PO). It is likely that the relative dose used for cats is even higher if the low oral bioavailability in humans is taken into account (30%; Xu et al., 2010). However, the same dose rate was effective for pain relief in rabbits and turtles (McMillan et al., 2008; Chapter 3). Indeed, it is similar to the injectable dose of tramadol tested in dogs (Giorgi et al., 2010). Since the side effects of TAP are dose-related in dogs (Giorgi et al., 2012a) and humans (Kleinert et al., 2008), a lower dose rate might reduce the adverse effects in cats.

The present study is the first pharmacokinetic study of TAP in cats, although the use of TAP in dogs has been recently considered (Giorgi, 2012). An initial pharmacokinetic study in dogs has suggested its prospective use as an injectable rather than oral drug, because of its very low oral bioavailability (Giorgi et al., 2012a). Another study by the same group has confirmed its efficacy as a pain reliever, as well as its good safety profile in rabbits after IV administration (Giorgi et al., 2013). In chapter 3, TAP produced the effective antinociception and the onset of the analgesic effect was rapid in turtles. To the authors' knowledge, there are no published reports of the pharmacokinetics and pharmacodynamics of this drug in cats thus far. Drug dosing schedules for the cat are often extrapolated from other species, despite evidence that cats metabolise some drugs uniquely (Boothe, 1990).

Both plasma concentrations and the pharmacokinetic data reported here show some inter- and intra-cat variation, in agreement with previous studies of opioids (Taylor et al., 2001). The use of cats of widely varying ages and both genders might have affected the results of our study. However, statistically significant associations between pharmacokinetic data and cat age or gender were not found, although a larger sample size might be helpful in verifying this point. Individual cat variability might be relevant in clinical practice where animals are often highly variable both in signalment, and

health. The plasma drug concentrations after IV administration were similar to those detected after the same IV dose in rabbits (Giorgi et al., 2013). Conversely, the period of TAP detection in plasma was longer than those reported for dogs (6 h; Giorgi et al., 2012a), rabbits (4 h; Giorgi et al., 2013) and goats (6 h; Chapter 2), despite the dose rates being lower than or equal to, respectively, the dose rate used in the present study. This is to be expected, as the $T_{1/2}\lambda z$ reported in this study was longer than those previously used for dogs, rabbits and goats (dogs, approximately 1 h, Giorgi et al., 2012a; rabbits, 0.52 h, Giorgi et al., 2013, 1.2 h, Chapter 2). Glucuronidation is the main metabolic pathway for TAP in humans, as 83% of an oral dose of TAP is converted to and excreted as an inactive glucuronidated metabolite. Cats have very limited UDP-glucuronyltransferase activity (Court and Greenblatt, 1997), which might explain the inter-species differences in $T_{1/2}\lambda z$ values. However, the elimination of half-life in turtles (4 h) was longer than that used for cats (Chapter 3). It might be explain that the metabolic rate of mammals is faster than that of reptiles (Berner, 1999).

The IM and SC bioavailability reported in this study was TAP in cats is relatively high and is in line with previous study of TAP in goats (Chapter 2). Moreover, this agrees with what is published for other classical (Barnhart et al., 2000; Taylor et al., 2001) and atypical (Giorgi et al., 2009) opioid drugs. Despite the lower mean C_{max} obtained after SC administration, the difference

in bioavailability compared to IM administration was not significant. Indeed, SC administration appeared to produce something similar to a depot effect, releasing the drug more slowly than after IM administration. This effect was especially appreciable in the pharmacokinetic curves from individual cats, which showed extended TAP elimination phases (although not significantly) and increased AUC values. This pharmacokinetic feature might also explain the lower number of adverse effects reported in the SC group.

In humans, the minimal effective concentration (MEC) is 0.67 μM/L, which is equivalent to 148 ng/mL (Tzschentke et al., 2007). If the human MEC was applied to the cat, the plasma drug concentration reported in the present study exceeded this value for over 3 h (Fig. 2). However, extrapolation of the MEC value from humans to animals might not be advisable and caution should be given (Giorgi and Yun, 2012). It is also recognized that there could be some discrepancy between plasma concentration and the actual effect at the receptor level (Toutain and Lees, 2004). Indeed, TAP was recently reported to be effective in rabbits for at least 10 h after a 5 mg/kg IV administration (Giorgi et al., 2013), although TAP concentrations were below the human MEC after 2 h, suggesting that TAP produces a long lasting effect.

The plasma concentration of TAP calculated after the simulation is exceeded for over 4.5 and 6 h, after 3 mg/kg TID and 5 mg/kg BID,

respectively. However, the dose regimen of TAP at 5 mg /kg BID is likely to produce more severe side effects than those reported in this study following a single injection, although the side effects in this study were transient. Administering TAP IM three times a day at 3 mg/kg might be a good compromise in terms of amount of drug administered and interval of administration. However, parameters such as onset time and time exceeding the MEC should be verified with further appropriately conducted pharmacokinetic/pharmacodynamic studies to clarify this.

References

1. Barnhart, M.D., Hubbell, J.A., Muir, W.W., Sams, R.A., Bednarski, R.M., 2000. Pharmacokinetics, pharmacodynamics, and analgesic effects of morphine after rectal, intramuscular, and intravenous administration in dogs. *American Journal of Veterinary Research* 61, 24-8.

2. Berner, N.J., 1999. Oxygen consumption by mitochondria from an endotherm and an ectotherm. *Comparative Biochemistry and Physiology Part B* 124, 25–31.

3. Boothe, D.M., 1990. Drug therapy in cats: Mechanisms and avoidance of adverse drug reactions. *Journal of the American Veterinary Medical Association* 196, 1297-1305.

4. Clutton, R.E., 2010. Opioid analgesia in horses. *Veterinary Clinics of North America: Equine Practice* 26, 493-514.

5. Court, M.H., Greenblatt, D.J., 1997. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. *Biochemical Pharmacology* 53, 1041-1047.

6. Etropolski, M., Kelly, K., Okamoto, A., Rauschkolb, C., 2011. Comparable efficacy and superior gastrointestinal tolerability (nausea, vomiting, constipation) of tapentadol compared with oxycodone hydrochloride. *Advances in Therapy* 28, 401-417.

7. Fox, M.S., 2010. Chronic pain in small animal medicine. Mason Publishing.

8. Gabrielsson, J., Weiner, D., 2002. Pharmacokinetic and pharmacodynamic data analysis: concepts and applications. Swedish Pharmaceutical Press.

9. Giorgi, M., 2008. Pharmacokinetic differences of tramadol in several animal species and human beings. *Journal of Veterinary Research* 63, 1-4.

10. Giorgi, M., 2012. Tramadol vs tapentadol: a new horizon in pain treatment? *American Journal of Animal and Veterinary Sciences* 7, 7-11.

11. Giorgi, M., Del Carlo, S., Łebkowska-Wieruszewska, B., Kowalski, C.J., Saccomanni, G., 2010. Pharmacokinetics of tramadol and metabolites after injective administrations in dogs. *Polish Journal of Veterinary Sciences* 13, 639-644.

12. Giorgi, M., Del Carlo, S., Saccomanni, G., Łebkowska-Wieruszewska, B., Turini, V., Kowalski, C., 2009. Biopharmaceutical profile of tramadol in the dog. *Veterinary Research Communications* 33, S189-192.

13. Giorgi, M., Meizler, A., Mills, P.C., 2012a. Pharmacokinetics of the novel atypical opioid tapentadol after oral and intravenous administration in dogs. *Veterinary Journal* 194, 309-313.

14. Giorgi, M., Meizler, A., Mills, P.C., 2012b. Detection and quantification of the novel opioid drug tapentadol in canine plasma by HPLC with spectrofluorimetric detection: development and validation of a new methodology. *Journal of Pharmaceutical and Biomedical Analysis* 67-68, 148-153.

15. Giorgi, M., Mills, P.C., Tayari, H., Rota, S., Breghi G., Briganti, A., 2013. Plasma concentrations of tapentadol and clinical evaluations of a combination of tapentadol plus sevoflurane for surgical anaesthesia and analgesia in rabbits (*oryctolagus cuniculus*) undergoing orchiectomy. *Israel Journal of Veterinary Medicine* 68, 141-148.

16. Giorgi, M., Yun, H., 2012. Pharmacokinetics of mirtazapine and its main metabolites in Beagle dogs: a pilot study. *Veterinary Journal* 192, 239-241.

17. Kleinert, R., Lange, C., Steup, A., Black, P., Goldberg, J., Desjardins, P., 2008. Single dose analgesic efficacy of tapentadol in postsurgical dental pain: the results of a randomized, double-blind, placebo-controlled study. *Anesthesia and Analgesia* 107, 2048-2055.

18. Kukanich, B., Papich, M., 2009. Opioid analgesic drugs. In: Veterinary Pharmacology and Therapeutics, Papich, M., Rivier J., (Eds), Wiley Blackwell.

19. Lascelles, B.D.X., Capner, C.A., Waterman-Pearson, A.E., 1999. Current British veterinary attitudes to perioperative analgesia for cats and small mammals. *Veterinary Record* 145, 601-604.

20. McMillan, C.J., Livingston, A., Clark, C.R., Dowling, P.M., Taylor, S.M., Duke, T., Terlinden, R., 2008. Pharmacokinetics of intravenous tramadol in dogs. *Canadian Journal of Veterinary Research* 72, 325-331.

21. Pascoe, P.J., 2000. Opioid analgesics. Veterinary Clinics of North America: Small Animal Practice 30, 757-772.

22. Raffa, R.B., Friderichs, E., Reimann, W., Shank, R.P., Codd, E.E., Vaught, J.L., 1992. Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. *Journal of Pharmacology and Experimental Therapeutics* 260, 275-285.

23. Taylor, P.M., Robertson, S.A., Dixon, M.J., Ruprah, M., Sear, J.W., Lascelles, B.D.X., Waters, C., Bloomfield, M., 2001. Morphine, pethidine and buprenorphine disposition in the cat. *Journal of Veterinary Pharmacology and Therapeutics* 24, 391-398.

24. Toutain, P.L., Lees, P., 2004. Integration and modelling of pharmacokinetic and pharmacodynamic data to optimize dosage regimens in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics* 27, 467-477.

25. Tzschentke, T.M., Christoph, T., Kögel, B., Schiene, K., Hennies, H.H., Englberger, W., Haurand, M., Jahnel, U., Cremers, T.I., Friderichs, E., De, Vry, J., 2007. (-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride (tapentadol HCl): a novel mu-opioid receptor agonist/norepinephrine reuptake inhibitor with broad-spectrum analgesic properties. *Journal of Pharmacology and Experimental Therapeutics* 323, 265-76.

26. Tzschentke, T.M., Jahnel, U., Kögel, B., Christoph, T., Englberger, W., De Vry, J., Schiene, K., Okamoto, A., Upmalis, D., Weber, H., Lange, C., Stegmann, J.U., Kleinert, R., 2009. Tapentadol hydrochloride: A next-generation, centrally acting analgesic with two mechanisms of action in a single molecule. *Drugs Today* 45, 483-496.

27. Vadivelu, N., Mitra, S., Hines, R.L., 2011. Peripheral opioid receptor agonists for analgesia: a comprehensive review. *Journal of Opioid Management* 7, 55-68.

28. Xu, X.S., Smit, J.W., Lin, R., Stuyckens, K., Terlinden, R., Nandy, P., 2010. Population pharmacokinetics of tapentadol immediate release (IR) in healthy subjects and patients with moderate or severe pain. *Clinical Pharmacokinetics* 49, 671-682.

Chapter 2. Pharmacokinetics of Tapentadol (TAP) after Intravenous (IV) and Intramuscular (IM) Administration in Goats (*Capra hircus*)

Abstract

The objective of the present study was to assess the pharmacokinetics of the novel atypical drug tapentadol (TAP) after intravenous (IV) and intramuscular (IM) injections in normal goats. A 2 x 2 cross over design study design was carried out. Each goat was given 5 mg/kg body weight of TAP by IV and IM routes. The concentrations of TAP in plasma were evaluated using HPLC. Transient adverse effects (tremors and ataxia) were noticed in some animals, especially after IV administration. The plasma concentrations vs. time course after the two administrations were fitted using a bi-compartmental model. After IM injection, TAP showed a very fast absorption (T_{max} = 0.17 h). The average volume of distribution and clearance after IV and IM administration were 4387 ± 1935 and 4076 ± 1082 mL/kg, and 4449 ± 1134 and 6328 ± 1351 mL h/kg, respectively. The IM bioavailability was quite high, despite being variable (87.78 ± 35.63%). In conclusion, TAP showed a short half-life, thus intravenous infusion rather than multiple daily administrations or a bolus might be more suitable in this animal species. However, it is premature to recommend the use of this drug in clinical practice.

I. Introduction

Opioids are widely used for treatment of pain in human and veterinary medicine, and are considered to be the prototypical class of analgesics (Fox, 2010). It is used in veterinary medicine not only for analgesia, but also in many other clinical applications (e.g. antitussive, antidiarrheal, emetic). A significant drawback of opioids is that they are generally controlled substances (Pascoe, 2000; Clutton, 2010) and have serious potential adverse effects (Vadivelu et al., 2011). Furthermore, prolonged treatment with many opioids may induce tolerance to their analgesic effects, meaning that long-term therapy with opioids must be carefully monitored and frequently increased to maintain a clinically satisfactory analgesic effect (Dickenson & Suzuki, 2005). It would therefore be useful to have alternative drugs available to control pain in animals.

The use of most opioid drugs in goats is off-label as most of these active ingredients are not approved for this species. Although extra-label use of approved drugs by veterinarians is common in the slightly less routine domestic species, there is minimal information on drug pharmacokinetics, which would be useful for determining appropriate drug dosages. For this reason, some opioid drugs such as tramadol (De Sousa et al., 2008), buprenorphine (Ingvast-Larsson et al., 2007) and methadone (Olsen et al., 2013) have been tested in goats. Tramadol and buprenorphine appear to be

unsuitable in this species because of the lack of production of the active metabolite (and hence efficacy) and the severe adverse effects, respectively. In regards to methadone, basic information only is available presently. Although it has a better safety profile compared to buprenorphine, it still retains some side effects and the short half-life, limiting its prospective use in combinational pain relief therapy (Olsen et al., 2013).

TAP is a novel analgesic opioid drug that is unusual in its possession of a dual mechanism of action (MOR agonist and NE reuptake inhibitor). As for tramadol, this feature makes the active ingredient an attractive potential progenitor of a new pharmacological class and a prospective drug for use in veterinary medicine (Giorgi, 2012).

TAP was launched on the European market for human use in 2011. TAP in humans has shown a lower incidence of adverse effects compared to equianalgesic doses of morphine (Kleinert et al., 2008) and oxycodone (Etropolski et al., 2011). This active ingredient has attracted the attention of the veterinary world because its MOR affinity is 50-fold lower than that of morphine but 120-fold higher that tramadol (Giorgi, 2012). Additionally, its second synergistic mechanism of action does not involve 5-HT reuptake, reducing the possibility of the "serotonin storm effect" reported following rapid IV tramadol injections. In brief; i) TAP is recommended in cases of moderate to severe pain (similar to morphine); ii) compared to morphine,

TAP produces much less nausea and vomiting and when these effects are present, their duration is shorter (Tzschentke et al., 2009); iii) this drug is not restricted/regulated in most European countries; iv) it does not require metabolic activation to be effective and variation in drug metabolism should not widely affect its efficacy.

Very few studies have been carried out in the veterinary field thus far. The pharmacokinetic features of TAP have been investigated in dogs after intravenous and oral administration, showing a very low oral bioavailability (4%) (Giorgi et al., 2012a). In rabbits undergoing castration, IV TAP was shown as having an excellent efficacy in reducing intra/post surgery pain (Giorgi et al., 2013). In cats, IV, IM and SC administration of TAP showed similar pharmacokinetic profiles (Chapter 1). In turtles, IM injection of TAP produced an effective antinociception against the thermal stimuli (Chapter 3). The side effects in these animal species have been reported as minor and transient.

The aim of the present research was to assess the pharmacokinetics of TAP after intravenous (IV) and intramuscular (IM) injection in normal goats.

II. Materials and Methods

1. Drugs and reagents

Pure powder (> 99.8% purity) of TAP hydrochloride was purchased from Bepharm Ltd. Pure powder (> 99.8% purity) of M1, used as internal standards (IS), was obtained from LCG Promochem. HPLC grade acetonitrile (ACN), dichloromethane (CH_2Cl_2) and diethyl ether (Et_2O) were purchased from Scharlau. Analytical grade acetic acid and sodium tetraborate decahydrate were obtained from BDH (Dublin, Ireland). HPLC grade water was obtained by distilling deionised water produced by a Milli-Q Millipore Water System (Millipore, Milan, Italy). All the other reagents and materials were of analytical grade and supplied from commercial sources. The injectable solutions were prepared by dissolving the pure TAP hydrochloride powder in saline to give a 5 mg/mL solution, which was then passed through a 0.45 μm filter, maintaining sterile conditions.

2. Animals

Six local Nubian dry non-pregnant female goats, aged between 3-8 years, with a body weight of 52-72 kg, were used. The goats were previously determined to be clinically healthy on physical examination, serum chemistry and haematological analyses. Animal care and handling was

performed according to the provision of the EC council Directive 86/609 EEC as well as according to Institutional Animal Care and Use directives issued by the Animal Welfare Committee of the Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem that approved the study protocol.

3. Experimental design

Goats were randomly assigned to two treatment groups. An open, single-dose, two-treatment, 2×2 cross over design was used. Two groups of goats (n = 3) were housed in open pens with dirt floors (3.5×3.5 m^2) at the Faculty facility. The drug was administrated in a single-dose fashion via either the IV (group I) or IM (group II) routes. All goats were fasted overnight before the experiment. In the first period, each goat in group I received a single IV dose of TAP solution (5 mg/mL) at 5 mg/kg injected slowly over 2 minutes into the left jugular vein. This dose was selected based on previous information describing the effectiveness of TAP in laboratory species (Giorgi et al., 2013). The other group (II) received a single IM injection of 5 mg/kg of TAP given into the rectus femoris portion of the quadriceps femoris muscle. An interval of 1 week (wash out period) was observed, to ensure complete metabolism and excretion of TAP. After this

period the groups were rotated and the experiment was repeated. By the end of the study each goat had received TAP by both administration routes.

A catheter was placed into the right jugular vein to facilitate blood sampling. Blood samples (2.5 mL) were collected at 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 10 h after administration of TAP, and placed in an ice bath in collection tubes containing lithium heparin. All blood samples were centrifuged within 30 min of collection, at 3,000×*g*, 4°C for 15 min to separate plasma. Harvested plasma was stored at -70 °C until analysis which occurred within 15 days of collection.

4. High performance liquid chromatography (HPLC)

The concentrations of TAP in plasma were evaluated using HPLC, according to the method previously described by Giorgi et al. (2012b). The analytical method was briefly re-validated in plasma from the goats. The HPLC system was an LC Waters (Waters, USA) consisting of quaternary gradient system (600 Controller, Waters, USA), in-line degasser (model AF, Waters, USA), photodiode array detector (2998 model, Waters, USA), multi lambda fluorescence detector (model 2475, Waters, USA) and autosampler (model 717 plus, Waters, USA). Data was processed using Empower ProTM software (Waters, USA). The chromatographic separation assay was performed with a SunFire C18 analytical column (150x4.6 mm inner

diameter, 5 μm particle size, Waters) 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 finally 5% B isocratically (25-32 min).

5. Preparation of plasma samples

Briefly, 50 μL of IS solution (0.5 μg/mL) and 0.2 mL 0.2 M borate buffer adjusted to pH 9.3 were added to a 1.5 mL polypropylene snap cap tube (Sarsedt, USA) containing 0.5 mL of plasma. After vortex-mixing, 0.4 mL of extraction solvent (Et_2O:CH_2Cl_2 7:3 v/v) was added, the tube was then vortexed (30 sec) and shaken for 5 min and then centrifuged for 10 min at 15,625×*g*. 0.3 mL of the organic layer was transferred into a clean 0.5 mL polypropylene snap cap conical tube, vortexed and shaken with 0.2 mL of back-extraction solvent (0.05 M HCl:ACN 1:1 v/v) for 5 min and centrifuged for 10 min at 15,625×*g*. The aqueous phase (50 μL) was injected onto the HPLC system.

6. Pharmacokinetic evaluation

The measured plasma concentrations of TAP were plotted versus time for each goat and data were analysed using a commercially available software program (Win Nonlin 5.3, Pharsight Corporation, Mountain View, CA, USA). For each data set, a compartmental approach was carried out (Gabrielsson & Weiner, 2002). Different models were assessed by visual inspection of the curve fits and the residuals' scatter plots, together with the goodness of fit measures incorporated in the software (including the Akaike and Schwartz criteria).

Maximum concentration (C_{max}) of TAP in plasma, and the time required to reach C_{max} (T_{max}) were predicted from the data. The concentration at time 0 (C_0) in the IV administration samples is estimated by back-extrapolating from the first two concentration values. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. The IM bioavailability (F%) was calculated from the ratio of the areas under the plasma TAP concentration curve, after IM and IV administration, indexed to their respective dose:

$$F\ (\%) = (AUC_{IM} \times Dose_{IV})/(AUC_{IV} \times Dose_{IM}) \times 100$$

7. Simulation of tapentadol (TAP) dosage regimens

Based on the pharmacokinetic analysis of pooled data, computer simulations (WinNonlin 5.3, Pharsight Corporation, Mountain View, CA, USA) were performed to determine intramuscular dosage regimens that maintain TAP plasma concentrations greater than the minimal effective concentration (MEC) in human (148 ng/mL) for roughly 50% of the dose interval.

8. Statistical analysis

Pharmacokinetic data were evaluated using the ANOVA test. Correlations between the value groups were carried out by Pearson's test. The results were presented as means ± standard deviation (SD). All analyses were conducted using GraphPad InStat (GraphPad Software). In all experiments, differences were considered significant if the associated probability level (*P*) was lower than 0.05.

III. Results

About three-four minutes after IV administration, some adverse effects including tremors and ataxia were noticed in two goats. However, they resolved rapidly (10 min) and spontaneously. These adverse effects were not detected after IM dosing. Three days after drug administration severe hair loss was noticed in goats from both groups. This effect was less intense in the group administered with IM injection.

1. Validation of bioanalytical method

The HPLC method was re-validated in the goat plasma. Briefly, TAP was linear (r^2 value > 0.98) in the range 5-5000 ng/mL. The intraday repeatability was measured as coefficient of variation and was lower than 8.2%, whereas accuracy, was lower than 6.1%.

2. Pharmacokinetics of tapentadol (TAP)

A bi-compartmental model best described the data set of all the animals in both groups. In both the groups, TAP concentrations were detectable in the plasma for up to 6 h. Some variability in drug plasma concentrations was detected among the subjects, especially in the terminal part of the curve. Fig. 1 and 2 report the single (A-F) and average TAP

plasma concentrations vs. time curves after the two administrations, respectively. After IM injection, TAP showed a very rapid absorption (T_{max} = 0.17 h). The average values of $T_{1/2\beta}$ after both administrations were quite different, but the large variability reported after the IV administration resulted in this difference being insignificant. In addition, Vd and Clt values were constant between the treatment groups. The average pharmacokinetic parameters calculated for the two administrations are reported in Table 1. The IM bioavailability (F%) was high, although variable, reaching average level of 88%.

3. Simulation of tapentadol (TAP) dosage regimen

A compartmental open pharmacokinetic model was fitted to the pooled data from six goats, and the model was used to simulate the concentration–time profile for several dosage regimens after IM administration (5 mg/kg BID; 2 mg/kg TID and 2.5 mg/kg TID). The best model included absorption term and biexponential decay.

Parameters of this average model were V1_F, K01, K10, K12 and K21 which were 3131 ± 543 (mL/kg), 49.04 ± 10.82 (1 /h), 1.84 ± 0.21 (1/h), 1.41 ± 0.36 (1/h) and 2.28 ± 0.56 (1/h), respectively. Following simulations of IM administration of TAP at a dose of 2.5 mg/kg q 8h, plasma concentrations were greater than the MEC value of 148 ng/mL for

approximately 1 h (Fig. 3). Simulation of IM administration of TAP at doses of 5 mg/kg BID gave drug concentrations exceeding the MEC for over 2 hours. This simulation was disregarded because the large dose of opioid drug could intensify the side effects reported in this study. Simulations with greater than three administrations per day were not attempted because of the associated difficulty in managing such regimes in non-hospitalized animals.

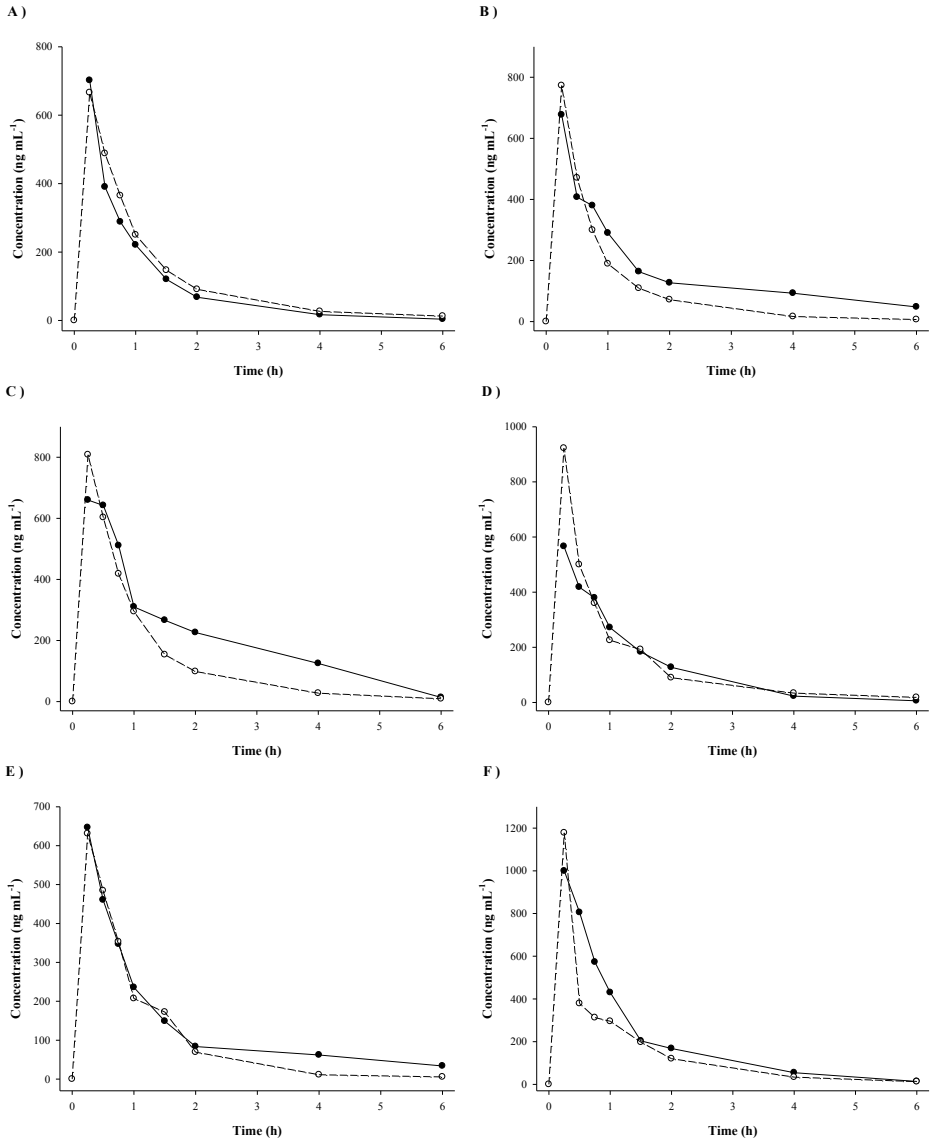


Fig. 1. Individual concentration vs. time curves (A-F) of tapentadol (TAP) (5 mg/kg) after single IV (-●-) and IM (--○--) administrations in goats.

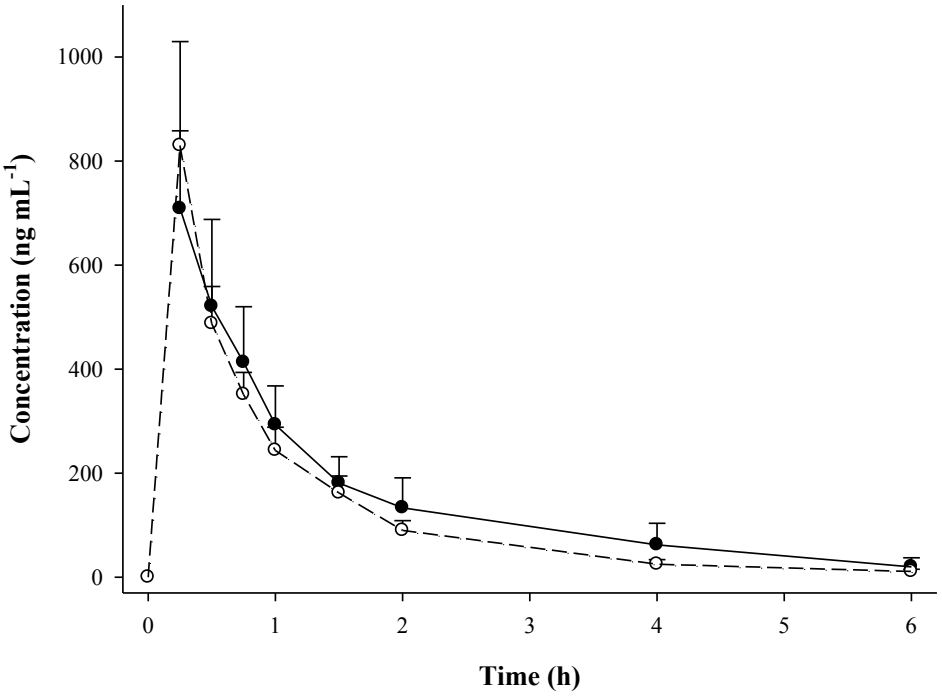


Fig. 2. Mean concentration vs. time curves of tapentadol (TAP) (5 mg/kg) after single IV (-●-) and IM (--○--) administrations in goats (n = 6).

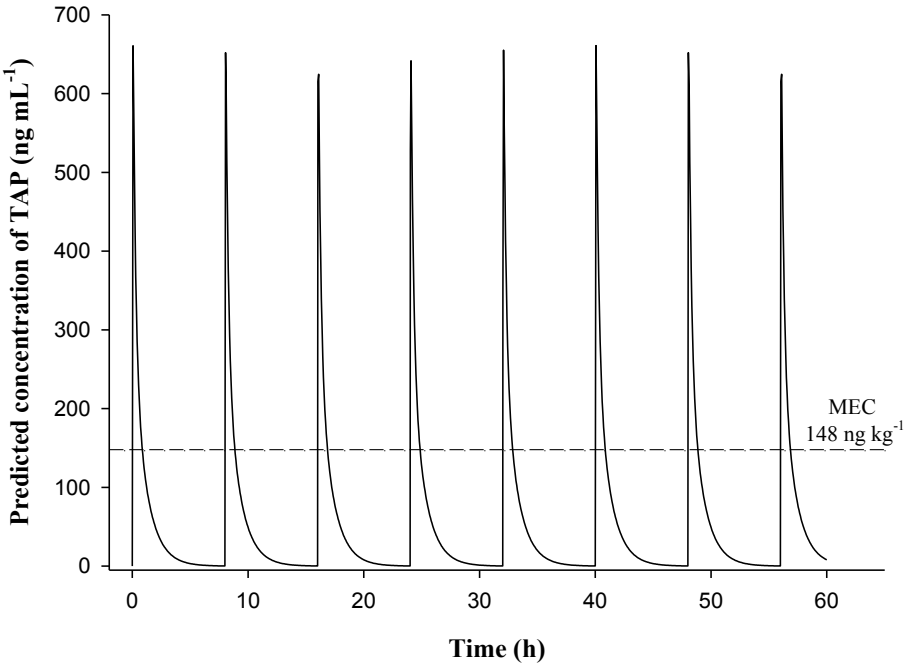


Fig. 3. Simulated average pharmacokinetic profile of plasma levels of tapentadol (TAP) after IM administration at 2.5 mg/kg TID in the goat. The horizontal dashed line indicates the MEC reported in humans (Tzschentke et al., 2007).

Table 1. Main average pharmacokinetic parameters after injection of tapentadol (TAP) at 5 mg/kg by IV, and IM route, respectively in goats (*n* = 6)

Pharmacokinetic Parameters (Unit)	IV		IM	
	Mean	SD	Mean	SD
A (ng/mL)	1639.74	2098.90	2682	3241
α (1/h)	3.80	4.90	3.13	1.52
B (ng/mL)	291	229	301	195
β (1/h)	0.44	0.39	0.61	0.23
k_{12} (1/h)	1.47	2.24	0.91	0.64
k_{21} (1/h)	0.99	0.90	1.21	0.74
k_{el} (1/h)	1.78	2.27	1.62	0.39
k_a (1/h)	NA	NA	17.30	14.37
$t_{1/2\alpha}$ (h)	0.37	0.20	0.27	0.14
$t_{1/2\beta}$ (h)	3.22	3.27	1.29	0.54
$t_{1/2ka}$ (h)	NA	NA	0.06	0.04
T_{max} (h)	NA	NA	0.17	0.07
C_{max} (ng/mL)	NA	NA	898	350
C_0 (ng/mL)	1931	2274	NA	NA
$AUC_{o-\infty}$ (ng/mL·h)	1186	298	804	123
V_d (mL/kg)	4387	1935	4076	1082
Cl_t (mL h/kg)	4449	1134	6328	1351
F (%)	NA	NA	87.78	35.63

A, intercept for the distribution phase; α, distribution slope; B, intercept for the elimination phase; β, elimination slope; k_{12}, rate of movement from compartment 1 to 2; k_{21}, rate of movement from compartment 2 to 1; k_{el}, rate of elimination; k_a, rate of absorption; $t_{1/2\alpha}$, the distribution half-life; $t_{1/2\beta}$, the elimination half-life; $t_{1/2ka}$, absorption half-life; T_{max}, time of peak concentration; C_{max}, peak plasma concentration; C_0, concentration at time 0; $AUC_{o-\infty}$, area under the curve from time zero to infinity; V_d, apparent volume of distribution of the area; Cl_t, total body clearance; F, bioavailability; NA, not applicable.

IV. Discussion

The side effects shown in this study were very similar to those previously reported in dogs administered with TAP (Giorgi et al., 2012a). The adverse effect profile concurs also with those reported in ruminant species after the administration of the atypical opioid tramadol (Giorgi et al., 2010; Cox et al., 2010). Side effects such as itching (due to histamine release), climbing, scratching, tail-flicking, and gnawing common in goats after administration of classical opioid drugs (Olsen et al., 2013; Ingvast-Larsson et al., 2007), were absent during the study. This difference might be due to the lower affinity of TAP at the MOR as compared to classical opioids (Giorgi, 2012). However, severe hair loss was noticed. As hair loss has never been reported in dogs, rabbits, cats and humans following TAP administration, it might be due to an unusual sensitivity of goats to this active ingredient. However, the pathogenesis of this response is unknown at this stage, further investigations are needed in small ruminant species to clarify this issue.

The present is the first pharmacokinetic study of TAP in goats. The use of TAP in veterinary medicine has been recently considered (Giorgi, 2012) and previous study has confirmed its efficacy as an analgesic, as well as its attractive safety profile in rabbits after IV administration (Giorgi et al., 2013). There is no data on the pharmacokinetics and pharmacodynamics of this

drug in ruminants thus far. Dosing schedules in the goat are often extrapolated from other species, however the extrapolation of pharmacokinetic profiles obtained in one species to another could be misleading (Szotáková et al., 2004).

Theoretically the rational for the use of TAP in ruminants that this active ingredient might bypass the shortcomings normally associated with other atypical opioids. Additionally, other reasons may be: (1) the full analgesic activity of TAP is contained within a single molecule (no enantiomers with different activities that could influence its analgesic and tolerability profile); (2) only the parent compound is involved in its pharmacological activity (i.e. no metabolic activation is necessary) (Terlinden et al., 2010); (3) the time dependent changes in the dynamic of opioid and monoaminergic analgesia occur in parallel (Schroder et al., 2011); (4) no CYP450 induction/inhibition exists which could negatively affect analgesia (Terlinden et al., 2007); (5) the 5HT reuptake inhibition triggering of adverse effects is negligible (Schiene et al., 2011).

Both, plasma concentrations and the pharmacokinetic data show some inter- and intra-subject variation, in line with previous studies on opioids (Ingvast-Larsson et al., 2007; Olsén et al., 2013). This variation might have been exacerbated by the wide age range of animals used in this study. However, the variations did not appear to be associated with age, although a

larger sample size of animals and inclusion of male animals would be needed to clarify this issue. This pattern might be relevant in clinical practice where patients are often highly variable both in signalment and health. The drug plasma concentration profile after IV administration was similar to that detected in dogs (Giorgi et al., 2012a). Conversely, the period of TAP detection in plasma was longer if compared to that reported in rabbits (4 h; Giorgi et al., 2013) administered IV with the same dose used in the present study. The IM bioavailability was large and it is in line with previous study of TAP in cats (Chapter 1). Moreover, this is in line with other IM bioavailabilities reported for both classical (Ingvast-Larsson et al., 2007) and atypical (Giorgi et al., 2010; Cox et al., 2010) opioid drugs.

In humans the minimal effective concentration (MEC) of TAP is reported to be 0.67 μM/L, which is equivalent to 148 ng/mL (Tzschentke et al., 2007). If the human MEC is assumed to apply also in the goat, the plasma drug concentration reported in the present study after IM or IV injection of TAP at 5mg/kg exceeds this value for over 2 hours. However, extrapolation of the MEC value from human to animals might not be completely advisable and caution should be used (Giorgi & Yun, 2012).

Multiple daily doses of TAP at 5 mg/kg administration are likely to produce more severe side effects than those reported in this study following a single injection. Additionally TAP is an opioid drug with an unknown safety

profile in goats and caution in dosing should be used. Administering TAP IM three times a day at 2.5 mg/kg gives plasma concentrations over the MEC for at least 1 h. This regime might be a good compromise in terms of amount of drug administered and interval of administration. Nevertheless, from the perspective of PK, 1 hour seems too short a time to maintain adequate plasma concentrations for pain relief. However, parameters such as MEC and efficacy should be verified with appropriate pharmacokinetic-pharmacodynamic studies, and further studies are needed to clarify this issue.

References

1. Clutton, R.E., 2010. Opioid analgesia in horses. *Veterinary Clinics of North America: Equine Practice* 26, 493-514.

2. Cox, S., Martin-Jimenez, T., van Amstel, S., Doherty, T., 2011. Pharmacokinetics of intravenous and intramuscular tramadol in llamas. *Journal of Veterinary Pharmacology and Therapeutics* 34, 259-264.

3. De Sousa, A.B., Santos, A.C.D., Schramm, S.G., Porta, V., Górniak, S.L., Florio, J.C., de Souza Spinosa, H., 2008. Pharmacokinetics of tramadol and o-desmethyltramadol in goats after intravenous and oral administration. *Journal of Veterinary Pharmacology and Therapeutics* 31, 45–51.

4. Dickenson, A.H., Suzuki, R., 2005. Opioids in neuropathic pain: Clues from animal studies. *European Journal of Pain* 9, 113-116.

5. Etropolski, M., Kelly, K., Okamoto, A., Rauschkolb C., 2011. Comparable efficacy and superior gastrointestinal tolerability (nausea, vomiting, constipation) of tapentadol compared with oxycodone hydrochloride. *Advances in Therapy* 28, 401-417.

6. Fox, M.S., 2010. Chronic pain in small animal medicine, Mason Publishing, London.

7. Gabrielsson, J., Weiner, D., 2002. Pharmacokinetic and pharmacodynamic data analysis: Concepts and applications, Swedish Pharmaceutical Press, Stockholm.

8. Giorgi, M., 2012. Tramadol vs tapentadol: a new horizon in pain treatment? *American Journal of Animal and Veterinary Sciences* 7, 7-11.

9. Giorgi, M., Meizler, A., Mills, P.C., 2012a.. Pharmacokinetics of the novel atypical opioid tapentadol after oral and intravenous administration in dogs. *Veterinary Journal* 194, 309-313.

10. Giorgi, M., Meizler, A., Mills, P.C., 2012b.. Detection and quantification of the novel opioid drug tapentadol in canine plasma by HPLC with spectrofluorimetric detection: development and validation of a new methodology. *Journal of Pharmaceutical and Biomedical Analysis* 67-68, 148-153.

11. Giorgi, M., Mills, P.C., Tayari, H., Rota, S, Breghi, G., Briganti, A., 2013. Plasma concentrations of tapentadol and clinical evaluations of a combination of tapentadol plus sevoflurane for surgical anaesthesia and analgesia in rabbits (*Oryctolagus Cuniculus*) undergoing orchiectomy. *Israel Journal of Veterinary Medicine* 68, 141-145.

12. Giorgi, M., Saccomanni, G., Del Carlo, S., Andreoni, V., 2010. Pharmacokinetic of tramadol and its major metabolites after intravenous and intramuscular injections in alpacas (*Vicugna pacos*). *Journal of Camel Practice and Research* 17, 123-126.

13. Ingvast-Larsson, C., Svartberg, K., Hydbring-Sandberg, E., Bondesson, U., Olsson, K., 2007. Clinical pharmacology of buprenorphine in healthy, lactating goats. *Journal of Veterinary Pharmacology and Therapeutics* 30, 249–256.

14. Kleinert, R., Lange, C., Steup, A., Black, P., Goldberg, J., Desjardins, P., 2008. Single dose analgesic efficacy of tapentadol in postsurgical dental pain: the results of a randomized, double-blind, placebo-controlled study. *Anesthesia and Analgesia* 107, 2048-2055.

15. Olsén, L., Olsson, K., Hydbring-Sandberg, E., Bondesson, U., Ingvast-Larsson, C., 2013. Methadone in healthy goats - pharmacokinetics, behaviour and blood pressure. *Research in Veterinary Science* 95, 231-237.

16. Pascoe, P.J., 2000. Opioid analgesics. Veterinary Clinics of North America: Small Animal Practice 30, 757-772.

17. Schiene, K., De Vry, J., Tzschentke, T.M., 2011. Antinociceptive and antihyperalgesic effects of tapentadol in animal models of inflammatory pain. *Journal of Pharmacology and Experimental Therapeutics* 339, 537-544.

18. Szotáková, B., Baliharová, V., Lamka, J., Nozinová, E., Wsól, V., Velík, J., Machala, M., Neca, J., Soucek, P., Susová, S., Skálová, L., 2004. Comparison of in vitro activities of biotransformation enzymes in pig, cattle, goat and sheep. *Research in Veterinary Science* 76, 43-51.

19. Terlinden, R., Kogel, B.Y., Englberger, W., Tzschentke, T.M., 2010. *In vitro* and *in vivo* characterization of tapentadol metabolites. *Methods & Findings in Experimental & Clinical Pharmacology* 32, 31-38.

20. Terlinden, R., Ossig, J., Fliegert, F., Lange, C., Göhler, K., 2007. Absorption, metabolism and excretion of ^{14}C-labeled tapentadol HCl in healthy male subjects. *European Journal of Drug Metabolism and Pharmacokinetics* 32, 163-169.

21. Tzschentke, T.M., Christoph, T., Kögel, B., Schiene, K., Hennies, H.H., Englberger, W., Haurand, M., Jahnel, U., Cremers, T.I., Friderichs, E., De Vry, J., 2007. (-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride (tapentadol HCl): a novel mu-opioid

receptor agonist/norepinephrine reuptake inhibitor with broad-spectrum analgesic properties. *Journal of Pharmacology and Experimental Therapeutics* 323, 265-76.

22. Tzschentke, T.M., Jahnel, U., Kögel, B., Christoph, T., Englberger, W., De Vry, J., Schiene, K., Okamoto, A., Upmalis, D., Weber, H., Lange, C., Stegmann, J.U., Kleinert, R., 2009. Tapentadol hydrochloride: A next-generation, centrally acting analgesic with two mechanisms of action in a single molecule. *Drugs of Today* 45, 483-496.

23. Vadivelu, N., Mitra, S., Hines, R.L., 2011. Peripheral opioid receptor agonists for analgesia: a comprehensive review. *Journal of Opioid Management* 7, 55-68.

Chapter 3. Pharmacokinetic/Pharmacodynamic Assessments of Tapentadol (TAP) in Yellow-Bellied Slider Turtles (*Trachemys scripta scripta*), After a Single Intramuscular (IM) Injection

Abstract

In reptiles, administration of opioid drugs has yielded unexpected results with respect to analgesia. Tapentadol (TAP) (Giorgi, 2012) is a novel atypical opioid drug labeled for human use. The aim of this study was to evaluate the pharmacokinetics and the pharmacodynamics of this drug in yellow-bellied slider, after a single intramuscular injection of 5 mg/kg of TAP. TAP plasma concentrations were determined by a validated HPLC-FL method, while an infrared thermal stimuli was applied to the plantar surface of the turtles' hind limbs to evaluate the thermal withdrawal latency (TWL) (Riviere et al., 1997).

TAP plasma concentrations were detectable between 1-24 h. The TAP treated group showed an increase in TWL 1 hour after drug administration (13.32 ± 6.40 s). Subsequently, TWL decreased with time, significant differences between treatment and control groups were apparent up to 10 h following treatment. A linear relationship (r^2 = 0.99) between TAP plasma concentration and effect was found. Given these findings, TAP appears to be

an attractive option for antinociception in turtles, due to its rapid onset and acceptable duration of effect.

I. Introduction

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 to determine, for the selected agent, a rational dosing regimen. Determining this is a long and complicated endeavour because of differences in the expression of enzymes, receptors and signal transduction molecules between species (Giorgi, 2012). 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 (Riviere et al., 1997). 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 years ago were considered wild animals are now pets and 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 (Giorgi and Owen, 2013; Giorgi et al., 2012b; Giorgi and Yun, 2012). 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 based on extrapolation from other species which have marked differences in their physiology (Riviere and Papich, 2013).

Opioids are considered the most effective drugs for controlling pain in mammals (Egger et al., 2013). In reptiles, opioid drug administration has yielded unexpected results with respect to analgesia. Butorphanol does not change thermal withdrawal latencies (TWL) in red-eared slider turtles (*Trachemys scripta elegans*) (Sladky et al., 2007) and bearded dragons (*Pogona vitticeps*) (Sladky et al., 2008) or thermal thresholds in green iguanas (*Iguana iguana*) (Fleming and Robertson, 2012). Buprenorphine did not alter responses to a noxious electrical stimulus administered to green iguanas (Greenacre et al., 2006) and did not provide an analgesic effect in red-eared slider exposed to a noxious thermal stimulus (Christoph et al., 2012). Morphine increased TWL in red-eared slider (Sladky et al., 2007) and bearded dragons (Sladky et al., 2008) at doses ranging between 1.5 and 20 mg/kg, but was ineffective at doses up to 40 mg/kg in corn snakes (Sladky et al., 2008). In contrast, the atypical opioid tramadol, whose use in mammals has been widely questioned (Giorgi et al., 2009a; 2009b; 2012c), has proven to provide antinociception (10 mg/kg SC) for at least 48 hours following administration in red-eared slider (Baker et al., 2011). Tramadol produces MOR activation (6000 times less than morphine) as well as inhibition of

serotonin (5HT) and norepinephrine (NE) reuptake in mammals. 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) (Raffa et al., 1992).

Tapentadol (TAP) is a novel atypical opioid drug labeled for human use. Based on 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, NE reuptake inhibitors (MORNRI) (Giorgi, 2012). Interestingly, even though its MOR affinity is 50-fold lower than that of morphine it has shown an equivalent analgesic activity. Additionally, after systemic administration in humans it is associated with a 2-3-fold reduction in the rate of adverse effects reported with oxycodone (Biondi et al., 2013). This finding, consistent across different pain relief evaluation models, may be due to a better brain penetration of TAP, but also suggests that the NE reuptake-inhibitory property, contributes to a more potent analgesia that would be expected solely from its MOR agonism (Tzschentke et al., 2006). If the reduction in adverse effects observed in humans holds true in reptiles, TAP would be an interesting analgesic. The objective of this study is to begin studying this promising molecule by assessing the PK/PD in yellow-bellied slider, after a single intramuscular injection of TAP.

II. Materials and Methods

1. Drugs and reagents

TAP hydrochloride was supplied as a pure powder (> 99.8% purity; Bepharm). 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 (CH_2Cl_2) and diethyl ether (Et_2O) were used in the assays (Scharlau, USA). Acetic acid and sodium tetraborate decahydrate (BDH, Ireland) were of analytical grade. HPLC grade water was obtained by distilling deionized water produced by a Milli-Q Millipore water system (EDM Millipore, Italy). All the other reagents and materials were of analytical grade and supplied from commercial sources. The injectable TAP solutions were freshly prepared by dissolving the pure TAP hydrochloride powder in saline to produce a 5 mg/mL solution, which was then passed through a 0.45 μm filter, maintaining sterile conditions.

2. Animals

Seven female and two male of turtles (*Trachemys scripta scripta*), with body weights ranging from 0.5 to 1.3 kg, supplied by a local park, were used for the study. Turtles were acclimated for a 2-week period prior to

commencement of the study. Turtles were judged to be in good health based on physical examination at the time of acquisition and at the start of the study, and through daily observation of behaviour and appetite. Specialized veterinary personnel (SR) made these observations. Turtles were divided according to the inclusion group (I or II) into two different 300 L plastic pools, 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 using an infrared lamp. Ambient temperature in the room varied from 25 to 26°C (electronic temperature sensors assured the constant temperature in both the water and basking area). Turtles were fed with a floating pelleted diet (a mix of fish and soy bean flour supplemented with vitamins and calcium chloride) three times per week. Animal care and handling was 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 Pisa University, which approved the study protocol (Protocol number 37070/2013).

3. Experimental design

Turtles were randomly assigned to two treatment groups, using slips of paper marked with the numbers 1-9, selected blinded from a box. A single-dose, single-treatment, unpaired, two-period crossover design was used.

Each turtle in group I (n = 5) received a single IM dose of TAP (5 mg/mL) at 5 mg/kg in the proximal front limb. This dose was selected based on previous information describing the effectiveness of TAP in rabbits (Giorgi et al., 2013). Group B (n = 4) received a single IM injection of saline (0.9% NaCl) (equivalent volume to opioid volumes) of TAP. A 1-month washout period was observed, to ensure complete metabolism and excretion of TAP. After this period, the groups were rotated and the experiment was repeated (second period). A fresh drug solution was prepared at this point. By the end of the study, each turtle had received both the saline and TAP treatment. Blood samples (1 mL) were collected from the subcarapacial venipuncture site at 0, 1, 2, 4, 6, 10, and 24, h after TAP administration and placed in collection tubes containing lithium heparin (MiniCollect, Greiner Bio-One). 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.

4. High performance liquid chromatography (HPLC)

Based on a previously published HPLC technique (Giorgi et al., 2012a), the analytical method was re-validated for turtle plasma samples. The HPLC system was an LC Waters (Waters, USA) consisting of quaternary gradient system (600 Controller, Waters, USA), in-line degasser (model AF, Waters, USA), photodiode array detector (2998 model, Waters, USA), multi lambda

fluorescence detector (model 2475, Waters, USA) and autosampler (model 717 plus, Waters, USA). Data was processed using Empower ProTM software (Waters, USA). The chromatographic separation assay was performed with a SunFire C18 analytical column (150 x 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).

5. 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, USA) containing 0.5 mL of plasma. After vortex-mixing, 0.4 mL of extraction solvent (Et_2O:CH_2Cl_2 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 onto the HPLC system.

6. Pharmacokinetic evaluation

The pharmacokinetic calculations were carried out using WinNonlin v 5.3 (Pharsight, USA). 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 ($T_{1/2}\lambda z$) was calculated using $T_{1/2} = 0.693/\lambda$. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. Changes in plasma concentration of TAP were evaluated using the standard non-compartmental analysis, and the relative pharmacokinetic parameters were determined using standard non-compartmental equations (Gabrielsson and Weiner, 2001).

7. Thermal antinociception 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 antinociception device (Hargreaves's instrument, model 37370, Ugo Basile) according to previously described methods (Baker et al., 2011; Sladky et al., 2007; 2008) with slight modifications. Turtles were gently dried using a smooth cloth and individually placed into clear, plastic boxes (300 × 200 × 150 mm, 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 upon 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 severe tissue damage. At each time point, the TWL was measured in one hind limb and then the other consecutively. When the difference between the two TWL values was > 2 s, a third measurement was obtained (at least 5 min after the last of the initial measurements). The observer in the analgesia experiments was blinded to treatments received. TWL were measured before drug administration (baseline) and at the same time as blood collections.

The thermal antinociceptive effect was expressed as percentage of Maximum Possible Response (% MPR) (Harris and Pierson, 1964), which was calculated as:

$$\%\ \text{MPR} = \frac{Ttest - Tcon}{Tcut - Tcon} \times 100$$

where *Ttest* represents TWL value after injection of TAP, *Tcon* is TWL value after injection of saline (control) and *Tcut* is the cut-off time (22.5 s).

8. Pharmacokinetic/pharmacodynamics (PK/PD) integration

The relation between the plasma concentration of TAP and % MPR was determined by sigmoid E_{max} model and the PD parameters were calculated from the model above. This model is described by the following equation (Riviere and Papich, 2013):

$$E = E_0 + \frac{(E_{max} \times C^n)}{(EC_{50}^n + C^n)}$$

where E is the effect (% MPR) at a specific concentration (C), E_0 is the effect when the concentration is 0, E_{max} is the maximum effect (% MPR), EC_{50} is the plasma concentration of TAP that results in 50% of maximum effect, C is the concentration of TAP in effect compartment and n is the Hill coefficient. The pharmacodynamic calculations were carried out using WinNonlin v 5.3 (Pharsight, USA).

9. Statistical analysis

Kolmogorov-Smirnov test was applied to verify data distribution. Pharmacodynamic data were evaluated using the two-way ANOVA (repeated-measures) to determine statistically significant differences between treatment and control values (cross over design). The TAP plasma concentrations and the pharmacokinetic and pharmacodynamic parameters

are presented as means ± standard error (SE). All analyses were conducted using GraphPad InStat (GraphPad Software). In all experiments, differences were considered significant if $P < 0.05$.

III. Results

One hour after the TAP IM administration, some signs of sedation were noticed in the animals. Turtles did not appear to be responsive to external stimuli (e.g. drying process) and had flaccid limbs and necks compared to the control animals. This effect was transient and was almost completely resolved at 2 h following drug administration.

1. Pharmacokinetic of tapentadol (TAP)

Average TAP plasma concentration vs. time curve after IM administration of 5 mg/kg in turtles is presented in Fig. 1. The plasma concentrations of TAP were in the range (37–1619 ng/mL) and detectable up to 24 h, except in three subjects. The corresponding pharmacokinetic parameters are shown in Table 1. The theoretical peak plasma drug concentration (C_{max}) of 1641 ± 749 ng/mL was observed at 1.22 ± 0.44 h (T_{max}) after injection. TAP was eliminated slowly in turtles with a long terminal half-life of 4.04 ± 2.10 h and it showed a large volume of distribution (Vz/F) of 4.30 ± 1.79 L/kg.

2. Pharmacodynamic of tapentadol (TAP)

Differences in TWL in control group animals (n = 9) were not statistically significant at any point tested. Hence, to establish the TWL baseline, all the saline solution data was grouped for each time point. No significant difference was found between control data obtained from the two study periods either. The T0 was 5.66 ± 0.92 s with average values of the whole base line ranging from 5.05 to 7.58 s (Fig 2a).

Animals given TAP showed an increase in TWL 1 hour after drug administration (13.32 ± 6.40 s). Subsequently, TWL decreased in proportion to time with significant differences from the saline group still apparent up to 10 h. The average TWL value in the TAP group after 24 h was 6.27 ± 1.22 s which is not significantly different than that of baseline (P = 0.18).

Mean MPR started at 1.69 ± 1.80 % (T0), increased to a maximum of 46.68 ± 12.30 % at 1 h and decreased to a minimum of 1.62 ± 2.77 % at 24 h (Fig. 2b). The MPR difference between TAP and saline group was still significant at 10 h.

3. Pharmacokinetic/pharmacodynamic (PK/PD) integration

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). Average plasma concentration associated with maximum % MPR of 46.68 ± 12.30 % was 1619 ± 242 ng/mL. Mean plasma concentration at each time point ranged between 37 ng/mL (24 h) and 1619 ng/mL (1 h), associated with % MPR of 1.62 and 46.68 %, respectively. A linear relationship (r^2 = 0.99) between TAP plasma concentration and % MPR was found (Fig. 3b).

PK/PD relation was evaluated with of use the values of % MPR associated with the plasma concentration of TAP using sigmoid E_{max} model. A value of the maximum antinociceptive effect (E_{max}) was 96.99 ± 7.13 % and the mean value of EC_{50} was 705 ng/mL. The PD parameters and the sigmoidal curve were displayed in Table 2 and Figure 4.

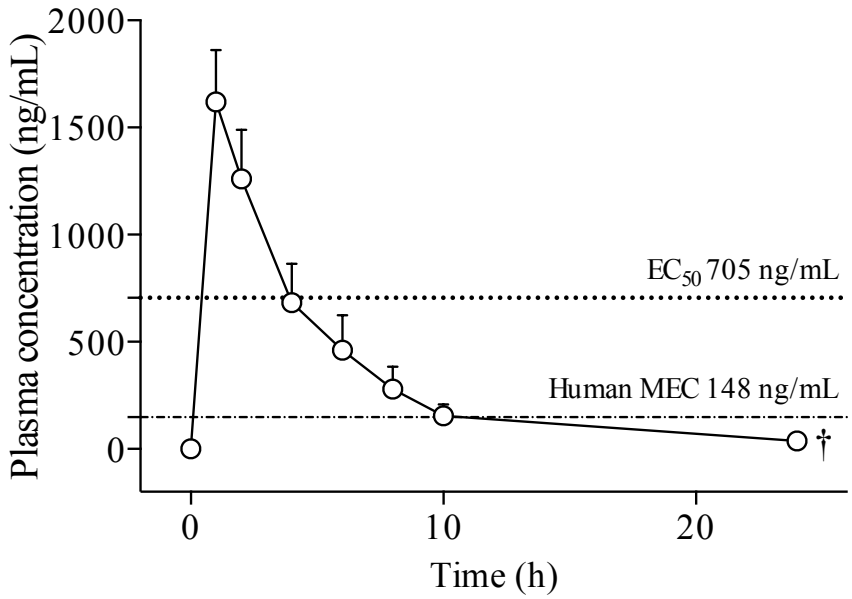


Fig.1. Mean plasma concentrations (+ SE) vs. time curve of tapentadol (TAP) after IM administration (proximal front limb) in turtles (n = 9). The dotted line represents the mean value of EC_{50} (705 ng/mL). The dashed and dotted line shows the MEC (148 ng/mL) reported for humans.

† Data obtained in six turtles.

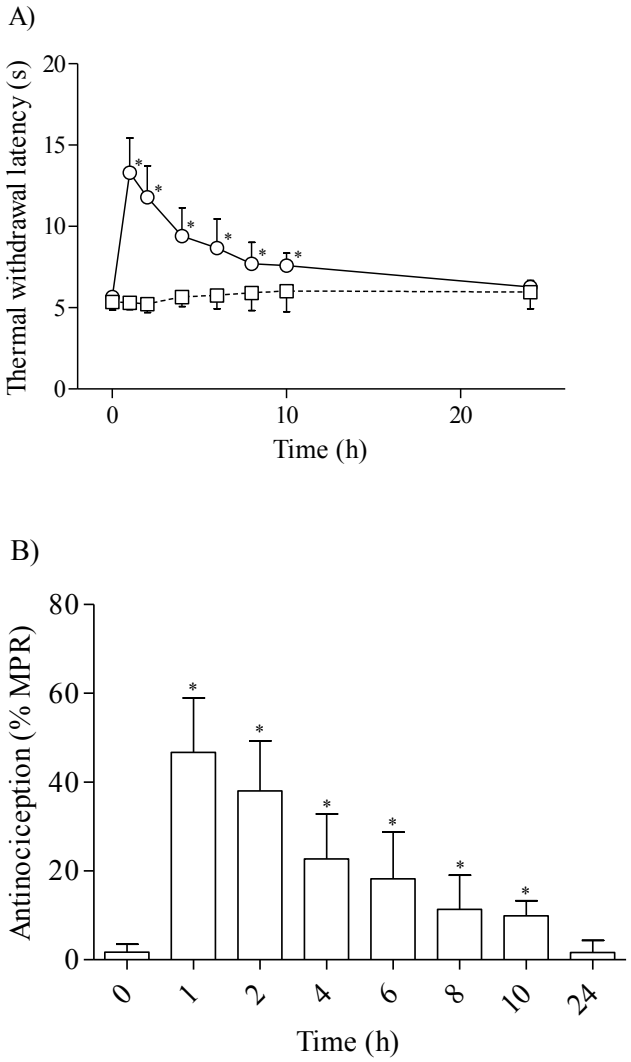


Fig. 2. (A) Mean (+ SE) TWL vs. time curve in turtles (n = 9) after IM saline (open square) and IM tapentadol (TAP) (open circle) administration (proximal front limb) (5 mg/kg); (B) mean (+ SE) % MPR after IM administration of TAP (5 mg/kg).

* Significantly different (P<0.05) from saline value (control).

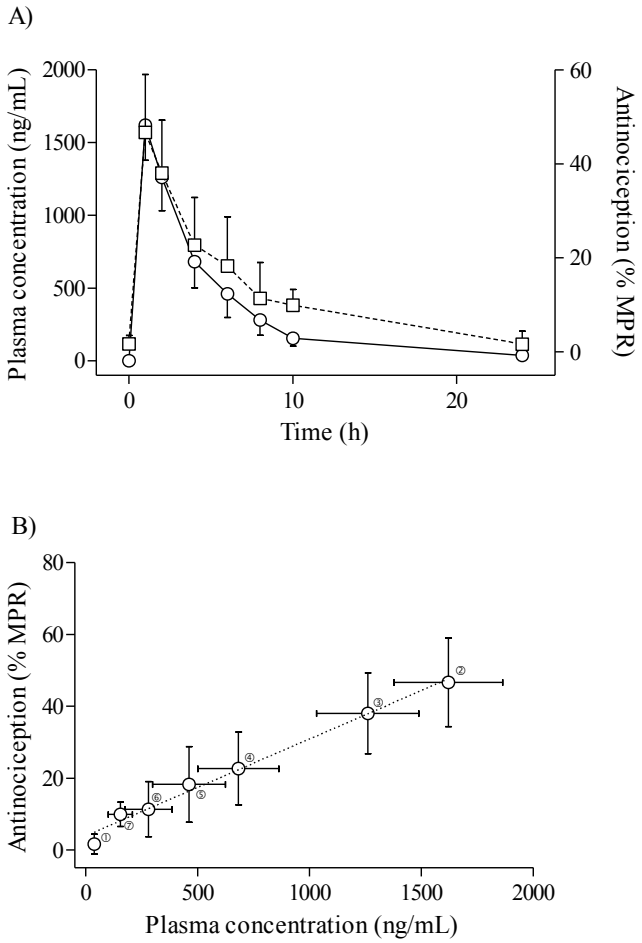


Fig. 3. (A) Mean (- SE) experimental plasma concentrations (open circles) of TAP and mean (+ SE) % MPR (open squares) vs. time curves in turtles (n = 9) after IM tapentadol (TAP) administration (proximal front limb) (5 mg/kg); (B) mean (± SE) experimental plasma concentrations (open circles) vs. mean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

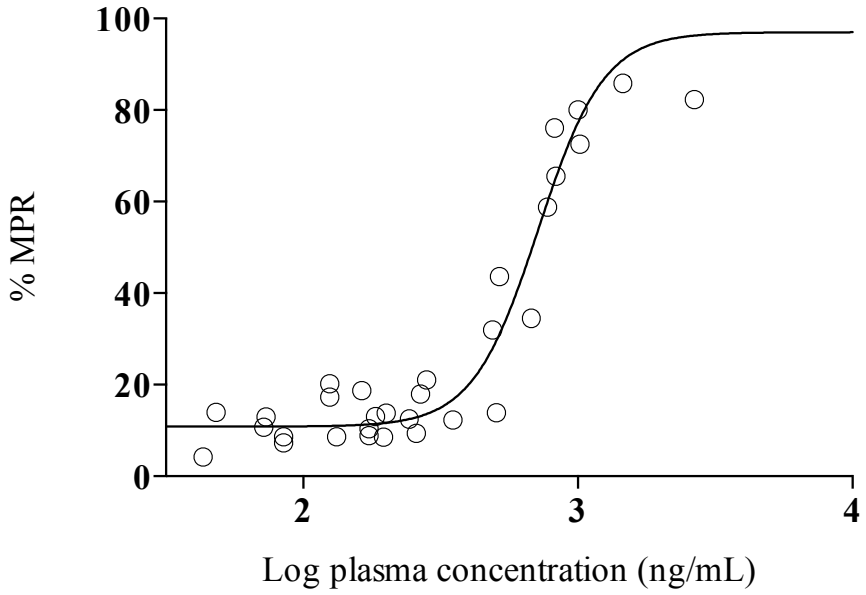


Fig. 4. Sigmoidal curve of tapentadol (TAP) plasma concentration vs. the antinociceptive effect (% MPR).

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

Pharmacokinetic parameters	Unit	Mean ±SE
λz	1/h	0.26±0.20
$T_{1/2}\lambda z$	h	4.04±2.10
T_{max}	h	1.22±0.44
C_{max}	ng/mL	1641±749
$AUC_{0-\infty}$	h ng/mL	7773±5751
Vz/F	L/kg	4.30±1.79
CL/F	L/min/kg	1.06±0.80
MRT	h	4.74±1.55

λz, first-order rate constant; $T_{1/2}\lambda z$, 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.

Table 2. Sigmoidal E_{max} model parameters of tapentadol (TAP) after IM injection (proximal front limb) of TAP at 5 mg/kg in turtles (*n* = 9)

Parameters	Unit	Mean ± SE
E_{max}	%	96.99 ± 7.13
E_0	%	5.92 ± 4.66
Log EC_{50}	ng/mL	2.85 ± 0.16

E_{max}, simulated maximum antinociceptive effecet of TAP, E_0, is the difference of % MPR value in the control, Log EC_{50}, log plasma concentration of TAP associated with half of the maximum antinociceptive effect. All data were obtained in six turtles.

IV. Discussion

If it is difficult to define and recognize whether an animal feels pain, it is even more challenging to objectively determine whether pain medication is effective in exotic animals. In general, to determine the efficacy of drugs in any species, it is important to determine the pharmacokinetic and pharmacodynamic properties of the drug in that species (Toutain and Lees, 2004). Knowing the pharmacokinetic values for a particular 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 duration of effect of analgesics (e.g. 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 species is not appropriate (Giorgi, 2012).

There is great potential for use of TAP in veterinary species (Giorgi, 2012). Its PK profiles have been already tested in dogs (Giorgi et al., 2012b), cats (Chapter 1) and goats (Chapter 2), and its PK/PD profile assessed in rabbits (Giorgi et al., 2013). The previous research has supported that the use of TAP in veterinary medicine may be suitable to pain control. However,

reptiles seem to react to opioids differently to mammals hence a PK/PD study in turtles is essential to understand the effectiveness of this drug. This study is the first PK/PD study of TAP in turtles using thermal stimulus pain model.

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. Due to these advantages, many nociceptive tests in red-eared slider (Baker et al., 2011; Sladky et al., 2007; 2009) have been carried out by this method. The TWL evaluated by Hargreaves's device has proven to be reproducible measure of complex nociceptive behaviour in rodents (Dirig et al., 1997) as well as other veterinary species (Kogel et al., 2014; SCHMID et al., 2010) and it has been extensively used for pain assessment in reptiles (Fleming and Robertson, 2012; Greenacre et al., 2006; Sladky et al., 2009; 2008; 2007). However, the acute thermal (anti-) nociception may be different from acute surgical (anti-) nociception and from longer-lasting pain like post-operative pain. For this reason, clinical studies are warranted to assess if TAP may or may not be useful in clinical settings at the dose studied here.

In an earlier pilot trial conducted on another group of turtles (to set up the experimental method), it was noticed that the amount of water residue on the turtles' limbs could affect the TWL measurement. This was in line with a previous study characterizing the variables in TWL measurement (Dirig et al., 1997). To avoid this issue, 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 the variability of the study. The potential influence of residue water on responses to thermal noxious stimuli should be considered in future studies.

In the present study, a different temperature (70° vs. 50° C) was set for the beam source compared to previous studies (Baker et al., 2011; Sladky et al., 2009; 2007). This variation was needed because the PK/PD design was contingent on the blood collection and TWL measurement occurring together. If the 50° C setting was used, a result could take several minutes, making the PK/PD protocol assumption invalid.

After IM injection of TAP, plasma drug concentrations were detectable up to 24 h. This persistence was longer than that reported in cats (8 h) (Lee et al., 2013) and goats (6 h) (Lavy et al., 2014) despite the same dose and route being used. TAP in turtles showed slower absorption (T_{max} = 1.22 h) than in cats (T_{max} = 0.25 h) (Lee et al., 2013) and goats (T_{max} = 0.17 h) (Lavy et al.,

2014). Furthermore, TAP in turtles reported a half-life almost twice that reported in cats ($T_{1/2}\lambda z$ 4.04 vs 2.28 h) (Lee et al., 2013). TAP is metabolized predominantly by glucuronidation in humans (Terlinden et al., 2007), 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 slower metabolic rate (Berner and Berner, 1999; Penick et al., 1998). These differences may have contributed to the long terminal half-life value of TAP found in turtles.

In a previous study, morphine (1.5 and 6.5 mg/kg SC) produced a thermal antinociception effect between 4 and 24 h and 2 and 24 h respectively, in red-eared slider (Sladky et al., 2007). Tramadol (10 mg/kg SC) produced a long lasting thermal antinociception effect between 6 and 48 h (Baker et al., 2011). According to Sladky et al (2009), thermal antinociception in response to opioids in red-eared slider appeared to be attributable mainly to MOR activation with a relatively minor contribution of delta-opioid receptor activation. It was assumed that the thermal antinociception effect was continued from 2 to 8 h after administration of an experimental MOR agonist ((D-Ala^2, N-Me-Phe^4, Gly^5-ol)-enkephalinacetate salt) by SC at a dose of 6.6 mg/kg (Sladky et al., 2009). 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. It is likely that onset of analgesic effect with TAP depends on the different administration route used (IM vs. SC). There is a possibility however, that the change of TWL is not solely the result of an antinociceptive effect. The sedation seen following the TAP administration might have affected the TWL values, especially at the initial measurements. A similar effect has been recently reported in American kestrels (*Falco sparverius*) (Ceulemans et al., 2014). Classical MOR agonists (e.g. morphine) cause a long lasting respiratory depression in red-eared slider (Sladky et al., 2007) because of their strong MOR activation. Unfortunately, respiratory rate was not evaluated in this study due to lack of the breathing chamber earlier described (Sladky et al., 2007). If MOR and MOR affinity are assumed to be similar across the species, it might expected that TAP in turtles causes less respiratory depression due to its lower MOR affinity compared to morphine and M1, as previously reported in humans (Tzschentke et al., 2006). Another variable that might have affected the TWL values is the effect of the observer. In the present study the turtles could see the investigators taking the observational data. This has been shown to be a variable in the response to noxious stimuli in iguanas (Fleming and Robertson, 2012). Further studies should be conducted to clarify whether this is an issue in turtles.

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 = 0.99) (Fig. 3b.); this varies from an earlier study reporting PK/PD of buprenorphine in cats (Robertson et al., 2005). The linear relationship between TAP plasma concentration and effect might be accounted for by the rapid blood-brain equilibration and its high MOR affinity (Tzschentke et al., 2007). This fits with the high lipophilicity of TAP (Fejős et al., 2014).

In this study, simulated value of E_{max} was 96.99 ± 7.13 % and it is in line with the maximum antinocicetive effect of TAP reported earlier in various pain models (Tzschentke et al., 2007). The mean value of EC_{50} in this study was 705 ng/mL and the TAP plasma concentration exceeded this value for over 4 h (Fig. 1). However, there were large variations in this value among turtles and the data-set from some turtles did not allow to apply the E_{max} model. Further PK/PD studies, using different pain model, would be needed to clarify these issues. Although the time above EC_{50} has lasted only 4 h, the thermal nociceptive behavior was significantly reduced at 10 h after TAP administration. It suggested that TAP produces a long lasting effect as described in previous study in the rabbit (Giorgi et al., 2013). Moreover, the TAP plasma concentration in turtles exceeded the human MEC from 1 to 10

h (Fig. 1b) and during this time, thermal antinociceptive effect was noted. However, extrapolation of the MEC value from humans to animals should be done with caution (Giorgi and Yun, 2012) and verified with larger sample size animal studies.

References

1. Baker, B.B., Sladky, K.K., Johnson, S.M., 2011. Evaluation of the analgesic effects of oral and subcutaneous tramadol administration in red-eared slider turtles. *Journal of the American Veterinary Medical Association* 238, 220–227.

2. Berner, N.J., Berner, N.J., 1999. Oxygen consumption by mitochondria from an endotherm and an ectotherm. *Comparative Biochemistry and Physiology Part B* 124, 25–31.

3. Biondi, D., Xiang, J., Benson, C., Etropolski, M., Moskovitz, B., Rauschkolb, C., 2013. Tapentadol immediate release versus oxycodone immediate release for treatment of acute low back pain. *Pain Physician* 16, E237–46.

4. Ceulemans, S.M., Guzman, D.S.M., Olsen, G.H., Beaufrère, H., Paul-Murphy, J.R., 2014. Evaluation of thermal antinociceptive effects after intramuscular administration of buprenorphine hydrochloride to American kestrels (*Falco sparverius*). *American Journal of Veterinary Research* 75, 705–710.

5. Christoph, M., Lesanna, L.L, Baker, B.B., Stephen, M.J., Kurt K.S., 2012. Antinociceptive efficacy of buprenorphine and hydromorphone in red-eared slider turtles (*Trachemys scripta elegans*). *Journal of Zoo and Wildlife Medicine* 43, 662–665.

6. Dirig, D.M., Salami, A., Rathbun, M.L., Ozaki, G.T., Yaksh, T.L., 1997. Characterization of variables defining hindpaw withdrawal latency evoked by radiant thermal stimuli. *Journal of Neuroscience Methods* 76, 183–191.

7. Egger, C.M., Love, L., Doherty, T., 2013. Pain Management in Veterinary Practice, 1st ed. John Wiley & Sons.

8. Fejős, I., He, Y., Völgyi, G., Kazsoki, A., Sun, J., Chen, W., Sohajda, T., Szente, L., Jiang, X., Béni, S., 2014. Tapentadol enantiomers: Synthesis, physico-chemical characterization and cyclodextrin interactions. *Journal of Pharmaceutical and Biomedical Analysis* 88, 594–601.

9. Fleming, G.J., Robertson, S.A., 2012. Assessments of thermal antinociceptive effects of butorphanol and human observer effect on quantitative evaluation of analgesia in green iguanas (*Iguana iguana*). *American Journal of Veterinary Research* 73, 1507–1511.

10. Giorgi, M., 2012. Veterinary Pharmacology: Is it Still Pharmacology's Cinderella? *Clinical and Experimental Pharmacology* 2, 2-2.

11. Gabrielsson, J., Weiner, D., 2001. Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Applications, Third Edition. CRC Press.

12. Giorgi, M., 2012. Tramadol Vs Tapentadol: Anew Horizon in Pain Treatment? *American Journal of Animal and Veterinary Sciences* 7, 7-11.

13. Giorgi, M., Del Carlo, S., Saccomanni, G., Łebkowska-Wieruszewska, B., Turini, V., Kowalski, C., 2009a. Biopharmaceutical profile of tramadol in the dog. *Veterinary Research Communications* 33, 189–192.

14. Giorgi, M., Del Carlo, S., Sgorbini, M., 2009b. Pharmacokinetics of tramadol and its metabolites M1, M2, and M5 in donkeys after intravenous and oral immediate release single-dose administration. *Journal of Equine Veterinary Science* 29, 569–574.

15. Giorgi, M., Meizler, A., Mills, P.C., 2012a. Quantification of tapentadol in canine plasma by HPLC with spectrofluorimetric detection: Development and validation of a new methodology. *Journal of Pharmaceutical and Biomedical Analysis* 67-68, 148–153.

16. Giorgi, M., Mills, P.C., Tayari, H., Rota, S., Breghi, G., 2013. Plasma concentrations of tapentadol and clinical evaluations of a combination of tapentadol plus sevoflurane for surgical anaesthesia and analgesia in rabbits (*Oryctolagus cuniculus*) undergoing orchiectomy. *Israel Journal of Veterinary Medicine* 68, 141-148.

17. Giorgi, M., Owen, H., 2013. Flupirtine: a human drug with potential for use in the veterinary field. *American Journal of Animal and Veterinary Sciences* 7, 213-217.

18. Giorgi, M., Saccomanni, G., Del Carlo, S., Manera, C., Lavy, E., 2012b. Pharmacokinetics of intravenous and intramuscular parecoxib in healthy Beagles. *The Veterinary Journal* 193, 246–250.

19. Giorgi, M., Saccomanni, G., Del Carlo, S., Mengozzi, G., 2012c. Pharmacokinetics of the tramadol injective formulations in alpacas (*Vicugna pacos)*. *Veterinary Science* 103–017.

20. Giorgi, M., Yun, H., 2012. Pharmacokinetics of mirtazapine and its main metabolites in Beagle dogs: a pilot study. *Veterinary Journal* 192, 239–241.

21. Greenacre, C., Takle, G., Schumacher, J., 2006. Comparative antinociception of morphine, butorphanol, and buprenorphine versus saline in the green iguana (*Iguana iguana*) using electrostimulation. *Journal of Herpetological Medicine and Surgery* 16, 88–92.

22. Harris, L.S., Pierson, A.K., 1964. Some narcotic antagonists in the benzomorphan series. *The Journal of Pharmacology and Experimental Therapeutics* 143, 141–148.

23. Kogel, B., Terlinden, R., Schneider, J., 2014. Characterisation of tramadol, morphine and tapentadol in an acute pain model in Beagle dogs. *Veterinary Anaesthesia and Analgesia* 41, 297–304.

24. Lavy, E., Lee, H.K., Mabjeesh, S.J., Sabastian, C., Baker, Y., Giorgi, M., 2014. Use of the novel atypical opioid tapentadol in goats (*Capra hircus*): pharmacokinetics after intravenous, and intramuscular administration. *Journal of Veterinary Pharmacology and Therapeutics* 37, 518–521.

25. Lee, H.K., Lebkowska-Wieruszewska, B., Kim, T.W., Kowaski, C.J., Giorgi, M., 2013. Pharmacokinetics of the novel atypical opioid tapentadol after intravenous, intramuscular and subcutaneous administration in cats. *Veterinary Journal* 198, 620–624.

26. Penick, D.N., Spotila, J.R., O'Connor, M.P., Steyermark, A.C., George, R.H., Salice, C.J., Paladino, F.V., 1998. Thermal independence of muscle tissue metabolism in the leatherback turtle, *Dermochelys coriacea*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 120, 399–403.

27. Raffa, R.B., Friderichs, E., Reimann, W., Shank, R.P., 1992. Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an "atypical" opioid analgesic. *Journal of Pharmacology and Experimental Therapeutics* 260, 275–285.

28. Riviere, J.E., Martin Jimenez, T., Sundlof, S.F., Craigmill, A.L., 1997. Interspecies allometric analysis of the comparative pharmacokinetics of

44 drugs across veterinary and laboratory animal species. *Journal of Veterinary Pharmacology and Therapeutics* 20, 453–463.

29. Riviere, J.E., Papich, M.G., 2013. Veterinary Pharmacology and Therapeutics. John Wiley & Sons.

30. Robertson, S.A., Lascelles, B.D.X., Taylor, P.M., Sear, J.W., 2005. PK-PD modeling of buprenorphine in cats: intravenous and oral transmucosal administration1. *Journal of Veterinary Pharmacology and Therapeutics* 28, 453–460.

31. Schmid, V.B., Seewald, W., Lees, P., King, J.N., 2010. *In vitro* and *ex vivo* inhibition of COX isoforms by robenacoxib in the cat: a comparative study. *Journal of Veterinary Pharmacology and Therapeutics* 33, 444–452.

32. Sladky, K.K., Kinney, M.E., Johnson, S.M., 2008. Analgesic efficacy of butorphanol and morphine in bearded dragons and corn snakes. *Journal of the American Veterinary Medical Association* 233, 267–273.

33. Sladky, K.K., Kinney, M.E., Johnson, S.M., 2009. Effects of opioid receptor activation on thermal antinociception in red-eared slider turtles (*Trachemys scripta*). *American Journal of Veterinary Research* 70, 1072–1078.

34. Sladky, K.K., Miletic, V., Paul-Murphy, J., Kinney, M.E., Dallwig, R.K., Johnson, S.M., 2007. Analgesic efficacy and respiratory effects of butorphanol and morphine in turtles. *Journal of the American Veterinary Medical Association* 230, 1356–1362.

35. Terlinden, R., Ossig, J., Fliegert, F., Lange, C., Göhler, K., 2007. Absorption, metabolism, and excretion of ^{14}C-labeled Tapentadol HCl in healthy male subjects. *European Journal of Drug Metabolism and Pharmacokinetics* 32, 163–169.

36. Toutain, P.L., Lees, P., 2004. Integration and modelling of pharmacokinetic and pharmacodynamic data to optimize dosage regimens in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics* 27, 467–477.

37. Tzschentke, T.M., Christoph, T., Kogel, B., Schiene, K., Hennies, H.H., Englberger, W., Haurand, M., Jahnel, U., Cremers, T.I.F.H., Friderichs, E., De Vry, J., 2007. (-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride (tapentadol HCl): a novel mu-opioid receptor agonist/norepinephrine reuptake inhibitor with broad-spectrum analgesic properties. *The Journal of Pharmacology and Experimental Therapeutics* 323, 265–276.

38. Tzschentke, T.M., De Vry, J., Terlinden, R., Hennies, H.H., Lange, C., Strassburger, W., Haurand, M., Kolb, J., Schneider, J., Buschmann, H., Finkam, M., Jahnel, U., Friderichs, E., 2006. Tapentadol hydrochloride. *Drugs of the Future* 31, 1053–1061.

Chapter 4. Synergistic Interaction of Tapentadol (TAP) and Flupirtine (FLP) in the Rat Orofacial Formalin Test

Abstract

Combination therapy with two or more analgesics is widely used in moderate and severe pain conditions. This combination of multiple analgesics with different modes of action can increase the analgesic effects and reduce side effects of each drug. The aim of this study is to evaluate the antinociceptive effect of tapentadol (TAP) and flupirtine (FLP) in rats, when administered separately or in combination, as well as their synergistic interaction.

After IP injection of TAP at different doses (2, 5, 10 and 15 mg/kg), the nociceptive behavior was reduced with dose-dependent manner in both phase I and II. Conversely, IP injection of FLP at different doses (0.6, 1.6, 3.3, 6.6 and 16.6 mg/kg) induced dose-dependent antinociceptive effect in phase II only. TAP was more potent and effective than FLP. The interaction between TAP and FLP were synergistic in phase II with an interaction index (γ) of 0.50 ± 0.24. The data reported in this study indicates that FLP enhances the antinociceptive effect of TAP and this drug combination is useful in the treatment of chronic pain.

I. Introduction

Opioids are widely used in treatment of moderate and severe pain in veterinary medicine. Indeed, they are the most effective class of drugs for controlling pain in veterinary medicine (Egger et al., 2013). However, persistent use of opioids in the relief of moderate and severe pain induces problem and unwanted side effects such as nausea, emesis, constipation and respiratory depression can be induced by long lasting treatment (Egger et al., 2013).

It is difficult to achieve effective pain control using a single analgesic due to development of tolerance and unwanted side effects. Combination therapy of different analgesic drugs offers an effective analgesia at reduced doses of individual agents, which may decrease the severity of the dose-related side effects (Playford et al., 1991; Raffa, 2001). Furthermore, combining agents with different modes of action may provide multimodal coverage of a broad spectrum of pain (Raffa, 2001). Several studies of combination of opioids with other classes have been demonstrated in various animal models (Abass et al., 2014; Argüelles et al., 2002; Díaz-Reval et al., 2010; Isiordia-Espinoza et al., 2011; Moreno-Rocha et al., 2012; Zhang et al., 2011).

Tapentadol (TAP) is an atypical opioid with dual mechanisms of action. Recently, it has been launched in the market for treatment of pain in humans.

TAP has received attention in the veterinary medicine due to its efficacy in pain control (Biondi et al., 2013; Etropolski et al., 2011; Schwartz et al., 2011; Vadivelu et al., 2011) and good safety profile (Imanaka et al., 2013; Lange et al., 2010; Schwartz et al., 2011) compared with classic opioids in human being. Recently, it has been tested in veterinary medicine, and a good profile of efficacy has been reported (Giorgi et al., 2013).

Flupirtine (FLP) is a centrally acting non-opioid analgesic that produces muscle relaxation (kumar et al., 2014). It is the first representative of the class of selective neuronal potassium channel openers (SNEPCO) (Aghajanian and VanderMaelen, 1982) and FLP facilitates the generation of the neuronal hyperpolarizing current (M-current). Thus, FLP decreases neuronal excitability by increasing the M-current (Kornhuber et al., 1999). FLP also inhibits the N-methyl-D-aspartate (NMDA) receptor indirectly (Singal et al., 2012). Furthermore, the muscle relaxant action of FLP can assist in the treatment of pain associated with spasticity and chronic musculoskeletal pain (Mueller-Schwefe, 2003; Wörz et al., 1995).

Molecules that inhibit the NMDA receptor are likely to have synergistic or additive effects with other analgesics, particularly opioids (Kolosov et al., 2012). Indeed, it has been demonstrated that the combination of FLP and opioids appear to have synergistic interactions (Capuano et al., 2011; Kolosov et al., 2012). However, there is no drug interaction study

between TAP and FLP. This study investigated whether combining FLP with TAP can enhance the antinociceptive effect in the orofacial formalin test more than either drug alone.

II. Materials and Methods

1. Drugs

TAP hydrochloride was supplied as a pure powder (> 99.8% purity) from Bepharm (Shanghai, China). Flupirtine was purchased as a commercial injection formula from Sigma Aldrich (St Louis, MI, USA). All drugs were diluted in sterile saline solution. The drug solutions were freshly prepared before experiments.

2. Animals

Male Wistar rats aged 7-8 weeks and weighing (200-220 g) were used. Animals were obtained from the Orient Bio Inc. (Gyeonggi-do, South Korea) and housed at 22°C on a 12 h dark-light cycle, with free access to food and water. Rats were judged to be in good health based on physical examination at the time of acquisition and at the start of the study, and through daily observation of behaviour and appetite. Before experiments, the animals were placed in the testing box for observation of behavior for at least 1 h in order to adapt to the environment. The animals were used only once and sacrificed using CO_2 after the experiment. All experiments were performed according to guidelines established by the Chungnam National University Institutional Animal Care and Use committee. This study was approved by the Local

Ethical Committee of Chungnam National University (Protocol No. CNU-00437).

3. Experimental design

Independent groups were used to describe the time course of the response for each drug. Groups for each drug (n = 6, each group) were received IP administration of saline or increasing doses of either TAP (2, 5, 10 and 15 mg/kg) or FLP (0.6, 1.6, 3.3, 6.6, and 16.6 mg/kg) of the same volume. Sterile saline was administered to control groups. Each experimental session included a control group to reduce variability of the result. After antinociception assessment of each drug, experimental ED_{30} values of each drug were determined. The values of ED_{30} add were calculated from the following equation (Tallarida, 2002):

$$ED_{30}\ add = \frac{ED_{30}\ flupiritine}{p_1 + Rp_2}$$

where ED_{30} flupirtine is the experimental ED_{30} of FLP, $p1$ and $p2$ are the proportions of each FLP and TAP in the total mixture, respectively, and R is the relative potency that is the ratio of ED_{30} of FLP alone to ED_{30} of TAP alone. Subsequently, a combination of TAP and FLP in a fixed ratio (1:1) was administrated IP at dose of ED_{30} add/2, ED_{30} add /4 and ED_{30} add. All drugs or saline were given 30 min before administration of 2.5% formalin.

4. Orofacial formalin test and antinociception assessment

The orofacial formalin test used in this study was previously described (Raboisson and Dallel, 2004). Briefly, 50 µl of 2.5% formalin solution diluted in isotonic saline was injected into the right upper lip subcutaneously, with a 31-gauge needle. After the injection, rats were placed in a glass chamber (30 x 30 x 30 cm) with mirrored sides for observation of behaviors. A video-camera was placed 1 m from the chamber and the behaviors of the rats were recorded for 45 min and the videos were analyzed using the JWatcher program developed by Dan Blumstein's Lab (University of California, Los Angeles) and the Animal Behaviour Lab (Macquarie University, Sydney). The recording video was divided into 15 blocks of 3 min and the number of seconds that the animals spend rubbing the injected site with the ipsilateral fore- or hindpaw was measured for each 3 min block. Time courses of the response to formalin for all drugs were determined as mean time of face rubbing up to 45 min. The nociceptive response induced by formalin is biphasic with the following phases; phase I was early and short-lasting (3-5 min) followed by a quiescent period (10-15 min); phase II was a prolonged (20-40 min) tonic phase (Raboisson and Dallel, 2004). In this study, the first 3 blocks (0-9 min) and 5 blocks (15-30 min) were

considered the first and second phase, respectively. The quiescent period (2 blocks, 9-15 min) was not included in the calculations.

The degree of nociception was assessed as the area under curve (AUC) of the time course of response (face rubbing). The AUC of both phases for each drug and combination were calculated by trapezoidal rule. Antinociceptive effect of both phases were established based on the percentage maximum possible effect (%MPE) calculated according to the following equation (Argüelles et al., 2002):

$$\%\ \mathrm{MPE}\ = \frac{(\mathrm{AUC\ vehicle}\ -\ \mathrm{AUC\ drug})}{\mathrm{AUC\ vehicle}} \times 100$$

where AUC vehicle represents the mean AUC of saline treatment groups and AUC drug represent the mean AUC of each drug treatment groups.

5. Isobolographic analysis

An isobolographic analysis was performed to evaluate the interaction between TAP and FLP according to the method previously described by (Tallarida, 2002). TAP and FLP showed different antinociceptive effects. As expected, TAP showed an antinociceptive effect both in phase I and II, while FLP was effective in phase II only. Thus, only the antinociceptive effect in phase II was used to calculate isobolographic analysis parameters.

First, the dose-response curves for phase II were examined and log dose-response curves were fitted using a non-linear regression analysis for the phase II of the orofacial formalin test. In this study, the maximum effect of FLP did not reach 50% of the total effect. The experimental ED_{30} values were used to determine ED_{30} add value for the combination study. Moreover, TAP and FLP showed a different maximum of the antinociceptive effect. The doses for FLP (less potent) were taken as equivalent dose of ED_{30} fractions of TAP (more potent) for IP administration. After administration of drug mixture at different doses (ED_{30} add, ED_{30} add/2 and ED_{30} add/4), the experimental ED_{30} (ED_{30} comb) was calculated from the dose–response curves of the combined drugs using standard linear regression analysis of log dose–response. The isobologram was constructed by connecting the ED_{30} of the FLP plotted on the abscissa with the ED_{30} of TAP plotted on the ordinate to obtain the additive line. The variance of ED_{30} add was evaluated according to the previous study (Tallarida, 2002).

The interaction index (γ) is a measure of the degree of synergism or sub-additivity. The interaction index (γ) was calculated as follows:

$$\gamma = ED_{30}\ \text{comb} / ED_{30}\ \text{add}$$

when the interaction index (γ) is close to 1, the interaction is additive. Values higher or lower than 1 indicates sub-additivity or synergism, respectively.

6. Statistical analysis

Dose-response data were evaluated using the one-way ANOVA (repeated-measures) to determine statistically significant differences between treatments and control values. Statistical significance between theoretical ED_{30} (ED_{30} add) and experimental ED_{30} (ED_{30} comb) was evaluated using Student's *t*-test. All analyses were conducted using GraphPad InStat (GraphPad Software) and Pharm tools pro trials (The McCary Group Inc.). All data were represented as mean ± standard error (SE). In all experiments, *P* values lower than 0.05 ($P < 0.05$) were considered significant.

III. Results

1. Antinociceptive effect of Flupirtine (FLP)

After injection of 2.5% diluted formalin solution, a typical pattern of face rubbing behavior was produced with two distinct phase; phase I, a phasic period lasting 10min after injection and a phase II, a tonic period due to sensitization mechanisms (Fig. 1). Time courses of response after IP administration of FLP in the range 0.6-16.6 mg/kg were presented in Fig. 1 and show a dose-dependent reduction in face rubbing behavior. Fig. 2 displays anitinociceptive effects (% MPE) of FLP in the range 0.6-16.6 mg/kg both in the first and second phase. In phase I, all tested doses of FLP failed to reduce nociceptive behavior (Fig. 1 and 2A). In phase II, FLP showed a dose-dependent reduction of nociceptive behavior and significantly increased % MPE in treatment groups at 3.3, 6.6 and 16.6 mg/kg compared to control (Fig. 2B). The mean MPE value in phase II increased to a maximum of 30.06 ± 8.31% in the 16.6 mg/kg FLP injection group.

2. Antinociceptive effect of tapentadol (TAP)

All doses of TAP that were tested reduced nociceptive behavior both in the first and second phases (Fig. 1B) in a dose-dependent manner. Particularly, in phase II, TAP (15 mg/kg) produced a greater antinociceptive

effect (% MPE) of 72.34 ± 2.81% than FLP (16.6 mg/kg, 30.06 ± 5.31%) (Fig. 3B). TAP significantly increased % MPE in groups with 5, 10 and 15 mg/kg injection compared to control (Fig. 3B) in both the first and second phases.

3. Isobolographic analysis for combination

After an administration of drugs mixture, nociceptive effects were reduced in a dose-dependent manner (data not shown).

Mean (± SE) values of the theoretical and experimental ED_{30} for combination were 8.34 ± 0.47 and 4.20 ± 1.21 mg/kg, respectively (Table 1). The ED_{30} comb value was significantly lower than the ED_{30} add value ($p<0.05$). Isobolographic analysis, using fixed ratio (1:1) ED_{30} fractions showed that combination of TAP and FLP produced antinociceptive effects greater than simple additivity (Fig. 4). The ED_{30} comb value was located in the region of the isobologram that indicates synergistic interaction (Fig. 4). The interaction index (γ) calculated in this study was 0.50 ± 0.24 that indicates synergistic interaction (Table 1).

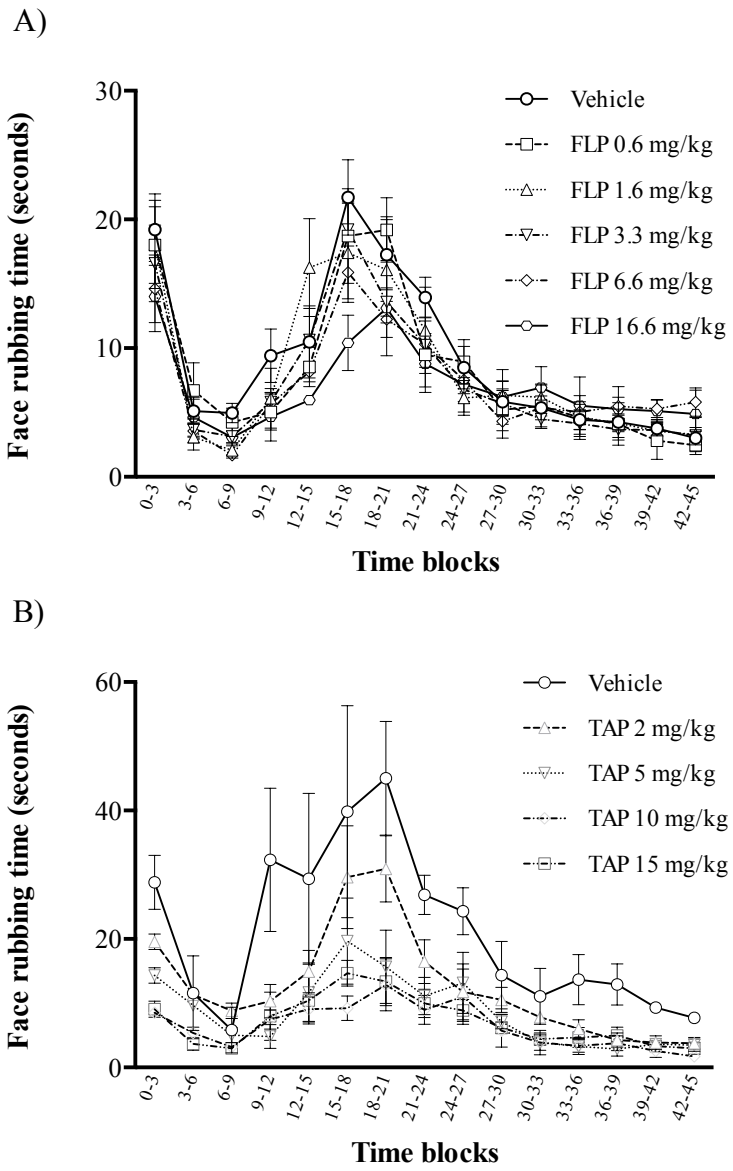


Fig. 1. Effect of flupirtine (A) and tapentadol (B) on the orofacial formalin test. Data represented as the mean time of face rubbing ± SE of rats (n = 6 for each group).

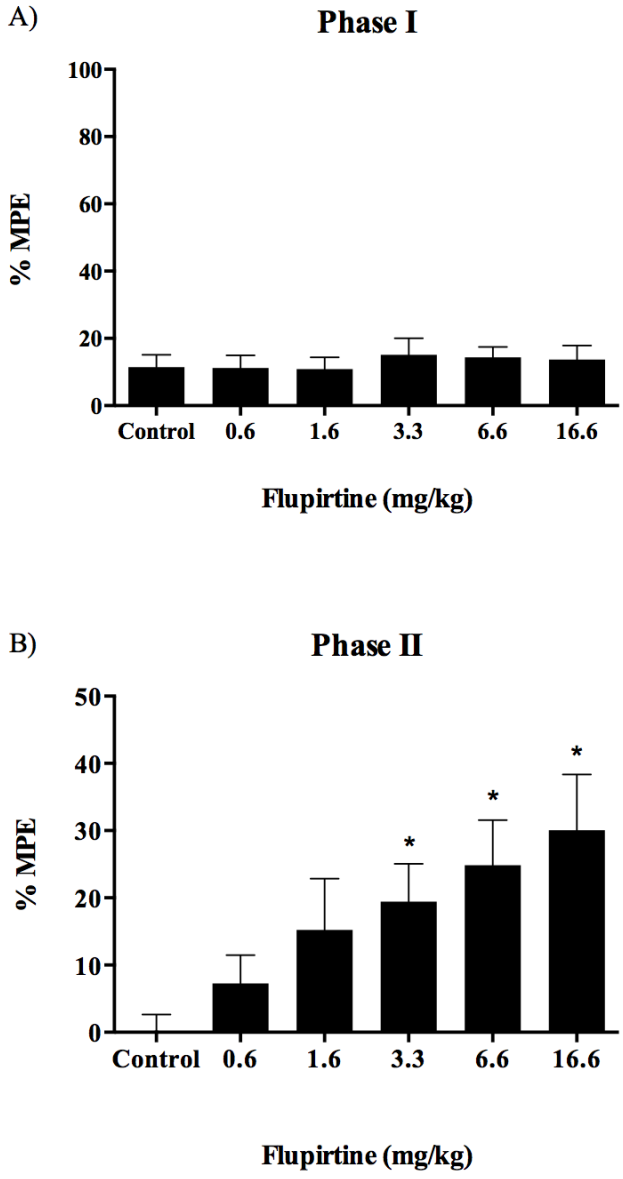


Fig. 2. Antinociceptive effect (% MPE) of flupirtine (FLP) with different doses in phase I (A) and phase II (B) of the orofacial formalin test. Data represented as mean % MPE ± SE of rats (n = 6 for each group). * $P < 0.05$ vs. controls.

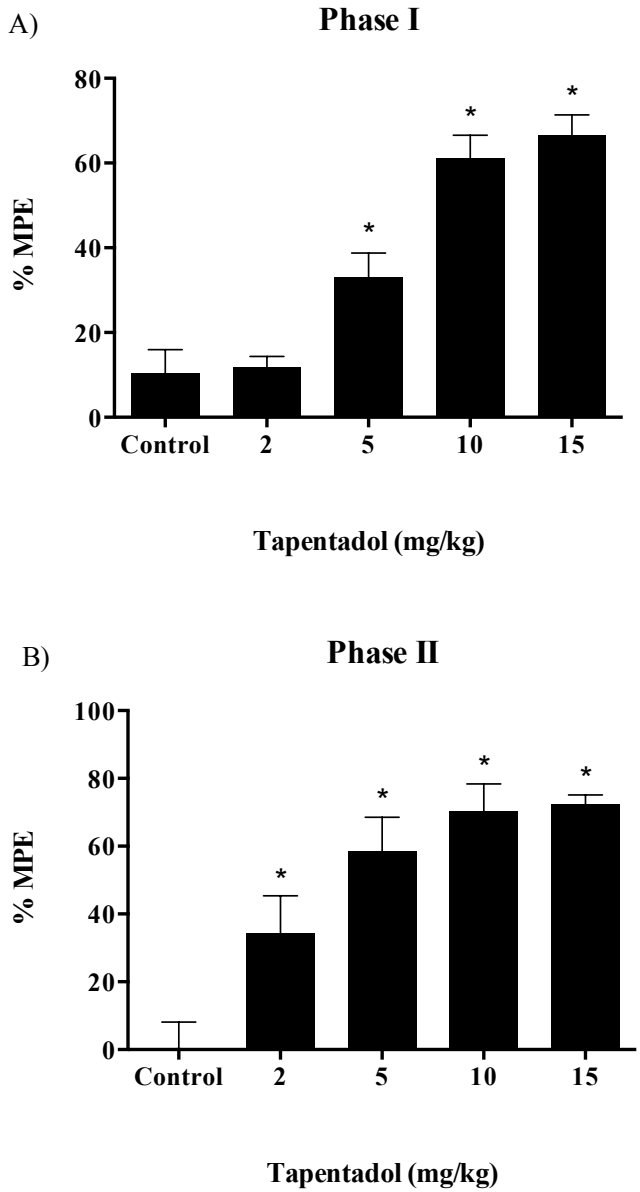


Fig. 3. Antinociceptive effect (% MPE) of tapentadol (TAP) with different doses in phase I (A) and phase II (B) of the orofacial formalin test. Data represented as mean % MPE ± SE of rats (n = 6 for each group). * $P < 0.05$ vs. controls.

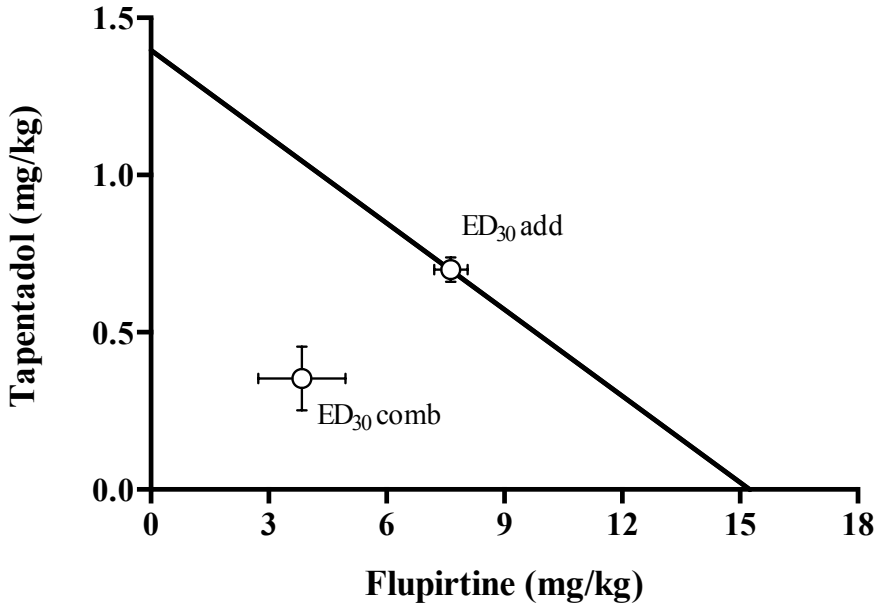


Fig. 4. Isobologram for combination of tapentadol (TAP) and flupirtine (FLP) in the orofacial formalin test. Data are represented as the means ± SE.

Table 1. Parameters of isobolographic analysis for the antinociceptive effect of combinations of tapentadol (TAP) and flupirtine (FLP)

Parameters	Drug combination (mg/kg) Mean ± SE
Theoretical (ED_{30} add)	8.34 ± 0.47
Experimental (ED_{30} comb)	4.20 ± 1.21*
Interaction Index (γ)	0.50 ± 0.24

* Significantly different (P<0.05) from ED_{30} add.

IV. Discussion

The orofacial formalin test is a recognized model of acute inflammatory nociception related to trigeminal pain. The injection of diluted formalin induces tissue injuries and generates behaviors (face rubbing) consisting in a biphasic response with a short-lasting first phase followed, after a quiescent period, by a second, long-lasting phase caused by inflammatory processes. The biphasic pattern of formalin induced nociception manifests different underlying mechanisms; the first phase is considered to be due to the direct chemical stimulation of nociceptive nerve endings (Dallel et al., 1995), while the second phase appears to be related to an inflammatory response with central sensitization (Hunskaar and Hole, 1987). Most of the methods commonly used for the study of nociception involve brief noxious stimuli such as noxious thermal (Falcon et al., 1996; Hargreaves et al., 1988) or mechanical (Ren, 1999; Rosenfeld et al., 1978) stimulation of facial skin, fore- and hind-paw and score thresholds or latencies of behavioral escape responses. Compared to these methods, the orofacial formalin test can avoid situations that animals turn their head and bite or lick the source of discomfort (Raboisson and Dallel, 2004). Indeed, the formalin induced models are the closest in nature to clinical pain (Le Bars et al., 2001).

The coadministration of a second agent with an opioid, which may be an analgesic or not, has following benefits: 1) to prolong analgesic duration, 2) to enhance analgesic efficacy, 3) diminish or minimize adverse effects, 4) to reduce opioid tolerance (Smith, 2008). Thus, multiple therapies of opioid and analgesics that enhance analgesic efficacy include combination with norepinephrine transporter modulators, nonsteroidal anti-inflammatory drugs (NSAIDs), local anesthetics, calcium channel blockers, cannabinoids, α_2-adrenergic agonists and $GABA_B$ agonists (Smith, 2008). Many of the interaction studies with opioid and analgesics have been reported in various nociception models (Abass et al., 2014; Capuano et al., 2009; Miranda et al., 2005; Zhang et al., 2011). In this study, the antinociceptive effect and synergistic interaction of the combination with TAP and FLP was evaluated by the orofacial formalin test. The results of this study demonstrated that the IP administration of TAP resulted in the dose-dependent antinociceptive effect in both phases of the orofacial formalin test. However, the IP administration of FLP alone reduced the nociceptive activity in the second phase only. In the second phase of the orofacial formalin test, TAP was superior to FLP both in terms of potency and efficacy. Indeed, the administration of the combination of TAP and FLP enhanced the antinociceptive effect more than that of TAP or FLP alone. Thus, the interaction between TAP and FLP was super-additive (e.g. synergism) with

an interaction index of 0.5. These results are similar to the previous interaction study of FLP in the orofacial formalin test (Capuano et al., 2011).

When FLP administered at doses of 3.3, 6.6 and 16.6 mg/kg, it produced the significant antinociceptive effect in the phase II. However, the administration of FLP at dose of 16.6 mg/kg (% MPR, 30.06%) did not produce the maximum antinociceptive effect. This value is lower than that reported in earlier study (10 mg/kg, > 45%) despite using of same animals and methods (Capuano et al., 2011). This difference might be attributed to the different formaline concentration used to induce nociceptive behavior (2.5 and 1.5% in this and Capuano et al., study, respectively). According to the Raboisson et al. (2004), it was reported that concentration-dependent nociceptive behavior is induced by formalin in a range of 0.5-2.5%. Therefore, the differences of nociception intensity might be related to the different concentrations of formalin. The concentration of formalin 2.5% was determined from an earlier pilot test because the concentration of 1.5% formalin was insufficient to induce the nociceptive behavior.

FLP is a non-opioid analgesic acting at the level of KCNQ channels for the treatment of a variety of pain states (Devulder, 2010). Drugs that modulate KCNQ channels may be useful in the treatment of pain. FLP has been investigated for its potential for use in veterinary medicine (Giorgi and Owen, 2013) and that FLP was found to be more effective than pentazocine

in post-surgery pain, but was comparable to pentazocine in cancer-related pain (Klawe and Maschke, 2009). FLP produced significant antinociceptive effects in the tail-flick test (Szelenyi et al., 1989) and was superior to tramadol for cancer-associated pain (Kolosov et al., 2012). However, its use has been limited to musculoskeletal pain due to side effects including dizziness, somnolence and cognitive impairment (Herrmann et al., 1993). FLP has become of interest again due to its indirect NMDA antagonism, even though there is no interaction with the binding sites of the NMDA receptor (Jakob and Krieglstein, 1997).

FLP significantly enhanced the antinociceptive effect of morphine in combination in the carrageenan paw inflammation and streptozocin-induced diabetic neuropathy pain models (Goodchild et al., 2008a). Moreover, synergistic interaction of the combination of FLP and tramadol, an atypical opioid, was observed in the orofacial formalin test (Capuano et al., 2011).

Previous studies showed that the combination of opioids and FLP produced a synergistic interaction (Capuano et al., 2011; Goodchild et al., 2008a; 2008b; Kolosov et al., 2012). Indeed, results reported in this study confirmed that FLP significantly increases the antinociceptive effect of TAP. FLP showed indirect NMDA receptor antagonism via activation of voltage independent potassium channels (Kornhuber et al., 1999). The generation of M-current is facilitated through the opening of potassium channels and the

opening of these channels which controls neuronal excitability (Orhan et al., 2012). Thus, the NMDA antagonism of FLP reduces hyperexcitation of the nociceptive neurons and it allows reduction in the therapeutic dose of opioids. Therefore, the co-administration of FLP with opioids can improve the efficacy of opioids and thus lower side effects by reduction of the doses required for the analgesic effect.

In this study, TAP produced an antinociceptive effect like that observed in previous combination studies (Schiene et al., 2011). Both of TAP and FLP showed an antinociceptive effect each and the combination of TAP and FLP displayed the synergistic interaction. In conclusion, the co-administration of FLP with TAP is regarded as useful in treatment of pain, because of its potential lower side effects if compared to classic opioids.

References

1. Abass, M., Mosbah, E., Rizk, A., Karrouf, G., 2014. Synergistic efficacy of tramadol and meloxicam on alleviation of pain and selected immunological variables after sciatic nerve ligation in rats. *International Journal of Veterinary Science and Medicine* 2, 14–20.

2. Aghajanian, G.K., VanderMaelen, C.P., 1982. α_2-adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: intracellular studies *in vivo*. *Science* 215, 1394–1396.

3. Argüelles, C.F., Torres-López, J.E., Granados-Soto, V., 2002. Peripheral antinociceptive action of morphine and the synergistic interaction with lamotrigine. *Anesthesiology* 96, 921–925.

4. Biondi, D., Xiang, J., Benson, C., Etropolski, M., Moskovitz, B., Rauschkolb, C., 2013. Tapentadol immediate release versus oxycodone immediate release for treatment of acute low back pain. *Pain Physician* 16, E237–46.

5. Capuano, A., De Corato, A., Treglia, M., Tringali, G., Navarra, P., 2011. Flupirtine antinociception in the rat orofacial formalin test: an analysis of combination therapies with morphine and tramadol. *Pharmacology, Biochemistry and Behavior* 97, 544–550.

6. Capuano, A., De Corato, A., Treglia, M., Tringali, G., Russo, Dello, C., Navarra, P., 2009. Antinociceptive activity of buprenorphine and lumiracoxib in the rat orofacial formalin test: a combination analysis study. *European Journal of Pharmacology* 605, 57–62.

7. Dallel, R., Raboisson, P., Clavelou, P., Saade, M., Woda, A., 1995. Evidence for a peripheral origin of the tonic nociceptive response to subcutaneous formalin. *Pain* 61, 11–16.

8. Devulder, P.D.J., 2010. Flupirtine in Pain Management. *CNS Drugs* 24, 867–881.

9. Díaz-Reval, M.I., Carrillo-Munguía, N., Martínez-Casas, M., González-Trujano, M.E., 2010. Tramadol and caffeine produce synergistic interactions on antinociception measured in a formalin model. *Pharmacology, Biochemistry and Behavior* 97, 357–362.

10. Egger, C.M., Love, L., Doherty, T., 2013. Pain Management in Veterinary Practice, 1st ed. John Wiley & Sons.

11. Etropolski, M., Kelly, K., Okamoto, A., Rauschkolb, C., 2011. Comparable efficacy and superior gastrointestinal tolerability (nausea, vomiting, constipation) of tapentadol compared with oxycodone hydrochloride. *Advances in Therapy* 28, 401–417.

12. Falcon, M., Guendellman, D., Stolberg, A., Frenk, H., Urca, G., 1996. Development of thermal nociception in rats. *Pain* 67, 203–208.

13. Mueller-Schwefe, G, 2003. Flupirtine in acute and chronic pain associated with muscle tenseness. *Fortschritte der Medizin Originalien* 121, 11–18.

14. Giorgi, M., Mills, P.C., Tayari, H., Rota, S., Breghi, G., 2013. Plasma concentrations of tapentadol and clinical evaluations of a combination of tapentadol plus sevoflurane for surgical anaesthesia and analgesia in

rabbits (*Oryctolagus cuniculus*) undergoing orchiectomy. *Israel Journal of Veterinary Medicine* 68, 141–148.

15. Giorgi, M., Owen, H., 2013. Flupirtine: a human drug with potential for use in the veterinary field. *American Journal of Animal and Veterinary Sciences* 7, 213-217.

16. Goodchild, C.S., Kolosov, A., Tucker, A.P., Cooke, I., 2008a. Combination therapy with flupirtine and opioid: studies in rat pain models. *Pain Medicine* 9, 928–938.

17. Goodchild, C.S., Nelson, J., Cooke, I., Ashby, M., Jackson, K., 2008b. Combination therapy with flupirtine and opioid: open-label case series in the treatment of neuropathic pain associated with cancer. *Pain Medicine* 9, 939–949.

18. Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77–88.

19. Herrmann, W.M., Hiersemenzel, R., Aigner, M., Lobisch, M., Riethmüller-Winzen, H., Michel, I., 1993. Long-term tolerance of flupirtine. Open multicenter study over one year. *Fortschritte der Medizin* 111, 266–270.

20. Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 30, 103–114.

21. Imanaka, K., Tominaga, Y., Etropolski, M., van Hove, I., Ohsaka, M., Wanibe, M., Hirose, K., Matsumura, T., 2013. Efficacy and safety of oral tapentadol extended release in Japanese and Korean patients with

moderate to severe, chronic malignant tumor-related pain. *Current Medical Research and Opinion* 29, 1399–1409.

22. Isiordia-Espinoza, M.A., Terán-Rosales, F., Reyes-García, G., Granados-Soto, V., 2011. Synergism between tramadol and meloxicam in the formalin test involves both opioidergic and serotonergic pathways. *Drug Development Research* 73, 43–50.

23. Jakob, R., Krieglstein, J., 1997. Influence of flupirtine on a G-protein coupled inwardly rectifying potassium current in hippocampal neurones. *British Journal of Pharmacology* 122, 1333–1338.

24. Klawe, C., Maschke, M., 2009. Flupirtine: pharmacology and clinical applications of a nonopioid analgesic and potentially neuroprotective compound. *Expert Opinion on Pharmacotherapy* 10, 1495–1500.

25. Kolosov, A., Goodchild, C.S., Williams, E.D., Cooke, I., 2012. Flupirtine enhances the anti-hyperalgesic effects of morphine in a rat model of prostate bone metastasis. *Pain Medicine* 13, 1444–1456.

26. Kornhuber, J., Bleich, S., Wiltfang, J., Maler, M., Parsons, C.G., 1999. Flupirtine shows functional NMDA receptor antagonism by enhancing Mg^{2+} block via activation of voltage independent potassium channels. *Journal of Neural Transmission* 106, 857–867.

27. Kumar, R., Keshri, U.P., Sharma, J., 2014. Flupirtine: a mini review. *Journal of Drug Delivery and Therapeutics* 3, 113–116.

28. Lange, B., Kuperwasser, B., Okamoto, A., Steup, A., Häufel, T., Ashworth, J., Etropolski, M., 2010. Efficacy and safety of tapentadol

prolonged release for chronic osteoarthritis pain and low back pain. *Advances in Therapy* 27, 381–399.

29. Le Bars, D., Gozariu, M., Cadden, S.W., 2001. animal models of nociception. *Pharmacological Reviews* 53, 597–652.

30. Miranda, H.F., Prieto, J.C., Pinardi, G., 2005. Spinal synergy between nonselective cyclooxygenase inhibitors and morphine antinociception in mice. *Brain Research* 1049, 165–170.

31. Moreno-Rocha, L.A., Domínguez-Ramírez, A.M., Cortés-Arroyo, A.R., Bravo, G., López-Muñoz, F.J., 2012. Antinociceptive effects of tramadol in co-administration with metamizol after single and repeated administrations in rats. *Pharmacology, Biochemistry and Behavior* 103, 1–5.

32. Orhan, G., Wuttke, T.V., Nies, A.T., Schwab, M., Lerche, H., 2012. Retigabine/Ezogabine, a KCNQ/K(V)7 channel opener: pharmacological and clinical data. *Expert Opinion on Pharmacotherapy* 13, 1807–1816.

33. Playford, R.J., Vesey, D.A., Haldane, S., Alison, M.R., Calam, J., 1991. Dose-dependent effects of fentanyl on indomethacin-induced gastric damage. *Digestion* 49, 198–203.

34. Raboisson, P., Dallel, R., 2004. The orofacial formalin test. *Neuroscience & Biobehavioral Reviews* 28, 219–226.

35. Raffa, R.B., 2001. Pharmacology of oral combination analgesics: rational therapy for pain. *Journal of Clinical Pharmacy and Therapeutics* 26, 257–264.

36. Ren, K., 1999. An improved method for assessing mechanical allodynia in the rat. *Physiology & Behavior* 67, 711–716.

37. Rosenfeld, J.P., Broton, J.G., Clavier, R.M., 1978. A reliable, facial nociception device for unrestrained, awake animals: Effects of morphine and trigeminal complex lesions. *Physiology & Behavior* 21, 287–290.

38. Schiene, K., De Vry, J., Tzschentke, T.M., 2011. Antinociceptive and antihyperalgesic effects of tapentadol in animal models of inflammatory pain. *Journal of Pharmacology and Experimental Therapeutics* 339, 537–544.

39. Schwartz, S., Etropolski, M., Shapiro, D.Y., Okamoto, A., Lange, R., Haeussler, J., Rauschkolb, C., 2011. Safety and efficacy of tapentadol ER in patients with painful diabetic peripheral neuropathy: results of a randomized-withdrawal, placebo-controlled trial. *Current Medical Research and Opinion* 27, 151–162.

40. Singal, R., Gupta, P., Nidhi, J., Gupta, S., 2012. Role of flupirtine in the treatment of pain-chemistry and its effects. *A Journal of Clinical Medicine* 7, 163–166.

41. Smith, H.S., 2008. Combination opioid analgesics. *Pain Physician* 11, 201–214.

42. Szelenyi, I., Nickel, B., Borbe, H.O., Brune, K., 1989. Mode of antinociceptive action of flupirtine in the rat. *British Journal of Pharmacology* 97, 835–842.

43. Tallarida, R.J., 2002. The interaction index: a measure of drug synergism. *Pain* 98, 163–168.

44. Tzschentke, T.M., De Vry, J., Terlinden, R., Hennies, H.H., 2006. Tapentadol hydrochloride. *Drugs of the Future* 31, 1053–1061.

45. Vadivelu, N., Timchenko, A., Huang, Y., Sinatra, R., 2011. Tapentadol extended-release for treatment of chronic pain: a review. *Journal of Pain Research* 4, 211–218.

46. Wörz, R., Lobisch, M., Schwittmann, B., 1995. Effectiveness of flupirtine in chronic tension headache. Results of a double-blind study versus placebo. *Fortschritte der Medizin* 113, 463-468.

47. Zhang, Y., Du, L., Pan, H., Li, L., Su, X., 2011. Enhanced analgesic effects of propacetamol and tramadol combination in rats and mice. *Biological and Pharmaceutical Bulletin* 34, 349–353.

General Conclusion

Tapentadol (TAP) is a novel opioid analgesic drug available for human medicine with peculiar mechanism of action and safety profile. It is also a promising molecule for pain relief for animals, even though having not yet been introduced in veterinary medicine. With this background in mind, basic and applied pharmacological investigations have been carried out in order to employ it as a pain killer in animals.

In these investigations, pharmacokinetic characterizations of TAP were evaluated in a wide range of animal species: the cat as a companion animal, the goats as a food producing animal, and the turtle as an exotic animal. Its pharmacodynamic profiles were also studied using the same animal species. Its PK/PD relationship was elucidated for its practical application in clinical setting. Drug interaction between TAP and FLP has been evaluated for their analgesic synergistic effect.

When TAP was administered intravenously, intramuscularly and subcutaneously in cats, its side effects were more severe and longer via IV administration if compared to IM and SC administrations. Adverse effects such as ataxia were noticed in goats given IV, as opposed to IM. On the other hand, pharmacokinetic parameters from IM administration appeared to be suitable to give reliable plasma concentrations of TAP. In addition, IM bioavailabilities in cats and goats were relatively high, in agreement with

high IM bioavailabilities reported for other opioids. From the viewpoint of its administration, IM dosing is regarded to be suitable in cats and goats. After IM administration of TAP, there were large interspecies variations in its half-lives and T_{max} between mammals (cats, goats) and reptiles (turtles). The average half-life value in turtles was approximately two times higher than those in cats and goats. TAP showed slower absorption and elimination in turtles as compared to cats and goats. Between the earlier mentioned two mammals, the half-life in cats was slightly longer than that in goats, however, other pharmacokinetic parameters were similar in these two animal species. It is hence postulated that its use needs some caution in turtles, particularly with liver and kidney dysfunction.

TAP produced excellent thermal antinociception in turtles. The thermal antinociceptive effect occurred rapidly and lasted as long as 10 hours. From the PK/PD study, a significant antinociceptive effect started to generate in above the plasma concentrations > 154 ng/mL assessed by the thermal stimuli test.

In this study, we had a scientific result that TAP exerted favorable analgesic effects in turtles for the first time, though still necessitating the elucidation of its safety profile before its active usage. Nevertheless, the results obtained in this work could pave the way for further research on its

potential use for reptiles in general. Needless to say, it can be an attractive option for antinociception in mammals as well.

Either TAP or FLP did produce antinociceptive effects in the orofacial formalin test after IP injection in rats. On the other hand, the combination of TAP and FLP resulted in a synergistic antinociceptive effect. Therefore, this co-administration is considered to enhance the antinociceptive effect of both drugs. The combination of TAP and FLP is hence proposed as a new combination to be tested in veterinary clinical trials.