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Pharmacokinetics of eugenol and its effects on thermal hypersensitivity in rats

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Abstract

Neuropathic pain is a type of chronic pain following central or peripheral nervous system lesions that cause allodynia (pain initiated by a non-painful stimulus) and hyperalgesia (increased pain sensation following a painful stimulus). The first objective of the study was to evaluate the pharmacokinetics of eugenol, the principle chemical constituent of clove oil, following a gavage administration (40 mg/kg) in male Sprague–Dawley rats. The second objective was to evaluate the effect of repeated oral administrations of eugenol on hyperalgesia and allodynia using an experimental model of neuropathic pain in rats. Thermal and mechanical sensitivity (Hargreave's test and von Frey filaments) were determined in sciatic nerve cuff-implanted rats. Sensitivities were assessed following repeated oral administrations of 40 mg/kg of eugenol or saline for 5 days ($n=6$ per group). Pharmacokinetic parameters were calculated using noncompartmental methods. Serial blood samples were collected over 24 h. Concentrations of eugenol in blood and plasma peaked rapidly following oral administration. Mean $T_{1/2}$ values of eugenol in plasma and blood were long (14.0 and 18.3 h, respectively), suggesting a potential accumulation of the drug following repeated administrations. Reaction time to thermal stimuli appeared to increase constantly following repeated administrations of eugenol. On the last day of treatment, eugenol treatments resulted in a statistically significant prolongation of the reaction time to thermal stimuli in rats compared to the saline group (Mean±S.E.M.: 11.4±1.23 vs. 6.1±0.53 s, $P<0.01$). These results support the hypothesis that eugenol may alleviate neuropathic pain and that the cumulative effect of the drug may be in part responsible for this effect following repeated daily administrations.

Keywords: Eugenol; Neuropathic pain; Hyperalgesia and allodynia; Sprague–Dawley rats

1. Introduction

Eugenol (2-methoxy-4-(2-propenyl) phenol), the principal chemical constituent of clove oil (*Eugenia aromatica*), has been used for decades in dentistry as an analgesic (Kozam, 1977; Ohkubo and Shibata, 1997). More recently, additional pharmacological properties of eugenol were demonstrated including anti-inflammatory, anti-bacterial, anesthetic and neuroprotective effects (Laekeman et al., 1990; Reddy and Lokesh, 1994; Wie et al., 1997; Guenette et al., 2006). Although the exact mechanism of action of its neuroprotective properties is still under study, *in vitro* research has shown an interaction of eugenol with vanilloid receptors with a potential inhibition of pain transmission by blocking this receptor (Yang et al., 2003). In addition, eugenol appears to interact with neurotransmitters involved in pain sensitivity, with an agonist effect on γ-aminobutyric acid (GABA) and an antagonist effect on NMDA (*N*-methyl-D-aspartate) glutamate receptors (Aoshima and Hamamoto, 1999; Yang et al., 2003), both of which play important roles in pain transmission.

Vanilloid receptors were first discovered by Thresh (1846) following a study on hot pepper burning sensation induced by capsaicine. Capsaicine burning sensation was induced by its specific interaction with a new kind of pain receptor, the capsaicine receptor, later named as the vanilloid receptor due to its structural similarity with vanilla (Nelson, 1919; Spath and

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Darling, 1930). More recent work has proven the importance of vanilloid receptors in nociception. Four subclasses (TRPV1, TRPV2, TRPV3 and TRPV4) of vanilloid TRP (transient receptor potential) channels have been established based on their specific activities (Caterina and Julius, 2001; Davis et al., 2000; Szallasi and Blumberg, 1999). TRPV1 is a non-selective cation channel receptor that functions as an integrator of painful chemical and physical stimuli including noxious heat and low pH. TRPV1 antagonists were shown to have analgesic effects on both inflammatory and neuropathic pain (Caterina et al., 1997; Ohkubo and Shibata, 1997; Tominaga et al., 1998). Only a limited number of substances have shown antagonistic activity for vanilloid receptors. Amongst these, capsaicin (Szallasi and Blumberg, 1999), resiniferatoxin (Szallasi and Blumberg, 1993), zingerone (Liu and Simon, 1996), scutigeral (Szallasi et al., 1999), olvanil (Lawand et al., 1997) and trialkylglycine (Garcia-Martinez et al., 2002) have been shown to be therapeutic candidates for treatment of neuropathic pain.

Neuropathic pain can be divided in two different pain types: hyperalgesia, which is an increase pain perception following painful stimuli, and allodynia which occurs secondary to an innocuous stimulus (Nichols et al., 1999). The resulting pain sensation is triggered by one or the other of these mechanisms, and can also switch from one to the other over time (Nichols et al., 1999). Persistent pain will usually evolve over time and spread to the contralateral parts of the body (GEDN, 2004). Neuropathic pain can be broadly defined as a result of nerve injury or malfunction in the peripheral or central nervous system (CNS) and is thought to arise via different origins such as amputation, trauma, metabolic pathologies, infectious pathologies, neoplasm and nutritional deficiencies (GEDN, 2004). Traditional pain killers (opioid analgesics) are usually ineffective against neuropathic pain (GEDN, 2004). This persistent chronic pain is characterised by the activation of specific receptors and the liberation of neuropeptides both locally (peripheral nerves) and centrally (Nichols et al., 1999). New therapeutic targets should therefore be evaluated for the treatment of this condition.

Due to its interaction with vanilloid receptors, and possibly other neurotransmitters involved in pain transmission, we propose to characterise the biodisposition *in vivo* of eugenol and its effects on pain sensitivity. The first objective of the study was to evaluate the pharmacokinetics of eugenol following a gavage administration in rats. The second objective was to evaluate the effect of repeated oral administrations of eugenol on hyperalgesia and allodynia using an experimental model of neuropathic pain in male Sprague–Dawley rats.

2. Materials and methods

2.1. Animals

A total of 24 male Sprague–Dawley rats (Charles River, St-Constant, QC, Canada) weighing 250–350 g were used for these experiments. Animals were housed in polycarbonate cages (Ancare, Bellmore, NY, U.S.A) on hardwood sawdust bedding (Beta chip, Northeastern Products Co., Warrenburg, NY, USA) and acclimated for 5 days prior to the initiation of the study. Rats received tap water and a standard laboratory rodent diet (Charles River Rodent chow 5075, St-Constant, QC, Canada) *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine of the University of Montreal prior to animal use and is in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Pharmacokinetics study

The pharmacokinetics of eugenol was assessed in plasma ($n=6$ rats) and red blood cells ($n=6$ rats) of intact rats. Following oral administration of eugenol (40 mg/kg), serial blood samples were collected from the jugular vein at predose, and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. For blood collections, animals were anesthetised with isoflurane (2%) for a short duration (approx. 1 min) prior to each blood collection. Plasma was collected following blood centrifugation for 10 min at 3200 ×*g*. Plasma and blood were stored at −80 °C pending the LC/MS/MS assay.

Eugenol mass balance between blood, plasma and RBC can be described by the following equation:

$$\mathrm{CONC_{BLOOD}} \times V_{\mathrm{BLOOD}} = \mathrm{CONC_{PLASMA}} \times V_{\mathrm{PLASMA}} + \mathrm{CONC_{RBC}} \times V_{\mathrm{RBC}} \quad (1)$$

where $\mathrm{CONC_{BLOOD}}$, $\mathrm{CONC_{PLASMA}}$ and $\mathrm{CONC_{RBC}}$ represent the concentrations of eugenol in blood, plasma, and RBC, respectively and V_{BLOOD}, V_{PLASMA} and V_{RBC} represent the volume of blood, plasma, and RBC, respectively. The volume occupied by RBC and plasma are related to hematocrit (H) and blood volume by the following equations:

$$V_{\mathrm{RBC}} = H \times V_{\mathrm{BLOOD}} \quad (2)$$

$$V_{\mathrm{PLASMA}} = (1-H) \times V_{\mathrm{BLOOD}} \quad (3)$$

By substituting Eqs. (2) and (3) in Eq. (1), eugenol concentrations in RBC were calculated with the following equation:

$$\mathrm{CONC_{RBC}} = [(\mathrm{CONC_{BLOOD}} - \mathrm{CONC_{PLASMA}}) \times (1-H)]/H \quad (4)$$

In this study, the hematocrit was fixed to a nominal value of 0.45 based on results found in the literature for male Sprague–Dawley rats (Matsuzawa et al., 1993).

2.3. Analytical assay

The analytical assay of eugenol in plasma and blood was performed according to methods previously published using dansyl chloride as a derivatizing agent (Beaudry et al., 2005). Briefly, eugenol and dansyl chloride were purchased from Sigma-Aldrich (St-Louis, MO, USA). Other chemicals, including acetonitrile, methanol, sodium hydroxide and formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). The HPLC system consisted of an autosampler Varian 9100 (Palo

Table 1
Summary of study design and procedures used in the experimental model of neuropathic pain in male Sprague–Dawley rats

Days	Procedures
1–5	Behavioural testing — Intact animals
5	Surgical procedures — Cuff implantation in right hind paw
6–7	Recovery period
8	Neuropathic pain model evaluation [a]
9	Neuropathic pain model evaluation [a]
10	Neuropathic pain model evaluation [a] — Baseline 1
11	Neuropathic pain model evaluation [a] — Baseline 2
12	Neuropathic pain model evaluation [a] — Baseline 3
13	Treatments and sensitivity assessment [a] — Day 1
14	Treatments and sensitivity assessment [a] — Day 2
15	Treatments and sensitivity assessment [a] — Day 3
16	Treatments and sensitivity assessment [a] — Day 4
17	Treatments and sensitivity assessment [a] — Day 5

[a] Thermal and mechanical sensitivity according to Hargreave's test and von Frey filaments, respectively.

Alto, CA, USA) and a Water 625 pump (Milford, MA, USA). The LC-MS/MS system used was a PESciex API 3^+ (Applied Biosystem/MDS Sciex, Concord, ON, Canada). Data was acquired on an Apple™ Macintosh® (Silicon Valley, CA, USA) equipped with operation system 7.4. Data acquisition and analysis were performed using MassChrom 1.0 (Concord, ON, Canada). Calibration curves were calculated from the equation $y=ax+b$, as determined by weighted ($1/x$) linear regression of the calibration line constructed from the peak-area ratios of the drug and the internal standard. A linear regression (weighted 1/concentration) was judged to produce the best fit for the concentration–detector relationship (Beaudry, 1999). The regression model used was determined using the sum of the squares of the deviation. By convention, the regression line is considered to properly fit the calibration set when the sum of squares of the deviation is minimized.

Eugenol was extracted from rat plasma or blood using protein precipitation. A total of 50 μL of sample was mixed with 250 μL of internal standard solution (250 ng/mL thymol in acetone) in a 1.5 mL centrifuge tube. The sample was then vortexed vigorously and the samples were allowed to rest 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately 12000 ×*g* for 10 min and 250 μL of the supernatant was transferred into a 650 μL injection vial. One hundred μL of dansyl chloride solution (1 mg/mL in acetone) and 20 μL of 100 mM NaOH solution were added to injection vials and the sample was heated at 60 °C for 10 min. The vial was vortex briefly and transferred to an autosampler for analysis. An isocratic mobile phase was used with a Keystone Scientific (Thermo) BDS Hypersil C8 50×2 mm column with a particle size of 5 μm. The mobile phase consisted of acetonitrile and 0.5% formic acid in water at a ratio of 80:20, respectively. The flow rate was fixed at 0.55 mL/min and eugenol eluted after 0.9 min and the internal standard (thymol) after 1.1 min. The eluent was split 1:10 prior to introduction into the electrospray source. Ten μL of the extracted sample was injected and the total run time was set to 2 min. The mass spectrometer was interfaced with the HPLC system using a pneumatic assisted electrospray ion source. The N_2 pressure of the nebuliser gas was set at 40 psi and the ESI electrode was set to 4000 V. The declaustering potential was set to 15 V and the collision energy (E_{lab}) to 25 V. The collision gas used was argon at 2.5×10^{14} molecules/cm^2. The SRM transition were m/z 398→171 and 384→171 for eugenol and thymol, respectively. The dwell time was set to 150 ms and the pause time at 5 ms.

2.4. Pharmacodynamic study

A summary of the study design using the experimental model of neuropathic pain in male Sprague–Dawley rats is presented in Table 1. Twelve rats were used for the pharmacodynamic study. Following 5 days of behavioural testing to obtain baseline values of thermal and mechanical sensitivity from intact animals, rats were implanted with a cuff around the right sciatic nerve to induce neuropathic pain. Aseptical surgical procedures were used in sciatic nerve cuff-implanted rats (Pitcher et al., 1999). Briefly, following anesthesia with isoflurane (AErrane; Baxter, Mississauga, ON, Canada), the right common sciatic nerve was exposed via blunt dissection at the level of the thigh (biceps femoris). A 2 mm PE-90 polyethylene cuff (Fischer Scientific Canada Inc., Nepean, ON, Canada) was placed around the nerve following its isolation using ball-tipped glass probes. The muscle was sutured with 3.0 vicryl suture and the skin was closed with a 2.0 silk suture.

Following a 2-day recovery period from surgery, thermal and mechanical sensitivities of the left (unoperated) and right (cuff-implanted) hind paws were determined using the Hargreave's test and von Frey filaments for the next 5 consecutive days to assess the efficacy of the experimental model to induce neuropathic pain. Briefly, a progressive light intensity, inducing heat, was applied on the left and right hind paws and animals were allowed to voluntarily retract their hind paw from the light source upon reaching the threshold of intolerable pain sensation (Hargreave's test). The maximum duration over which the hind paw was exposed was set to 20 s. Mechanical sensitivity was also evaluated on the left (unoperated) and right (cuff-implanted) hind paws using von Frey filaments based on a previously published method (Vachon et al., 2004). For the von Frey test, paw withdrawal thresholds were evaluated on the plantar surface of the right hind paw following surgery using von Frey monofilaments (0.4 g–50 g; Stoelting, Wood Dale, IL, USA). Monofilament application was to the central region of the plantar surface and the hairs were applied until buckling occurred. Values reported are the threshold force (*g*) necessary to elicit the mechanical withdrawal of the hind paw. The threshold was taken as the lowest force that caused at least 3 withdrawals out of 5 consecutive stimuli.

Rats were then distributed in two groups to receive either daily oral doses (40 mg/kg) of eugenol ($n=6$) or a saline solution ($n=6$) for the next 5 consecutive days. To avoid bias, rats were distributed in two groups in order to obtain similar mean thermal sensitivity levels (Hargreave's test) prior to treatment administration. The eugenol solution (50 mg/mL) consisted of 0.5 mL eugenol, 0.5 mL cremophor, 1 mL ethanol (99%) and 8 mL saline 0.9%. For the next 5 consecutive days, thermal hyperalgesia and allodynia tests were performed within 30 min following oral gavage of eugenol or the saline solution.

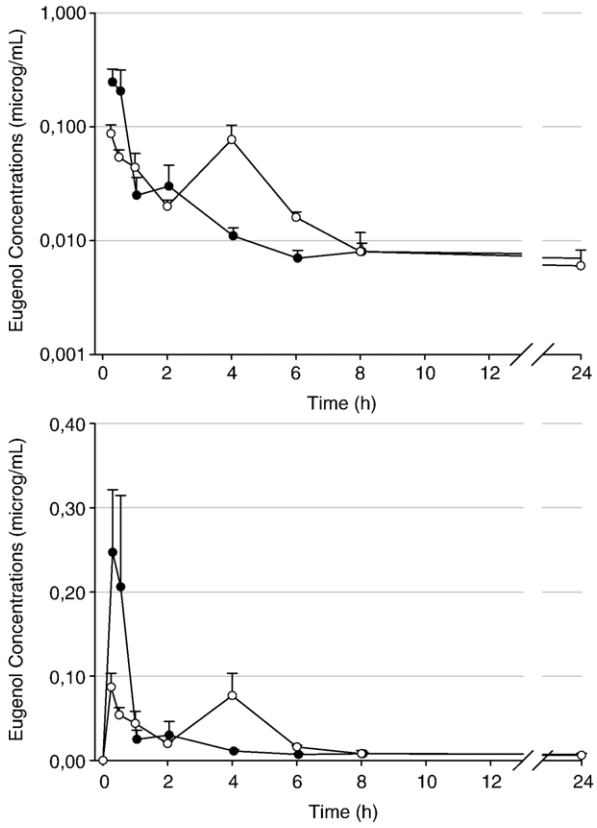

Fig. 1. Mean (±S.E.M.) concentrations of eugenol in blood (full circles) ($n=6$) and plasma (empty circles) ($n=6$) following a single oral administration (40 mg/kg) in male Sprague–Dawley rats on semi-log and linear scale (top and bottom panels respectively).

2.5. Pharmacokinetic and pharmacodynamic parameters

The following pharmacokinetic parameters were calculated in intact animals: area under the curve from time zero to the last detectable concentration (AUC_{0-t}) using the linear trapezoidal rule; area under the curve extrapolated to infinity (AUC_{0-inf}); maximum observed concentration (C_{max}); time of maximum concentration (T_{max}); apparent first-order terminal elimination rate constant (Kel); the terminal elimination half-life ($T_{1/2}$ calculated as 0.693/kel); and the apparent oral clearance (CL/F, calculated as Dose/AUC_{0-inf}) (Rowland and Tozer, 1995). Descriptive statistics were calculated for all pharmacokinetic parameters. All pharmacokinetic parameters were calculated using Kinetica® Version 4.1.1 (InnaPhase Corporation). Statistical analyses were performed using SYSTAT 8.0 for Windows (SPSS Inc.).

In order to evaluate the efficacy of the experimental model to induce neuropathic pain, thermal sensitivity results measured during the behavioral test period (Day 1–5) were compared to those observed during the neuropathic pain period (Day 8–12). Individual values of thermal sensitivities were measured in the morning of Day 1–5 and Day 8–12. Paired *t*-test analyses were performed between the pooled data collected during the behavioral test period (Day 1–5) and during the neuropathic pain evaluation period (Day 8–12). Individual values of thermal and mechanical sensitivities measured over the last 3 days of the neuropathic pain evaluation period (Day 10–12) were used to calculate mean baseline results of thermal and mechanical sensitivities for each rat. Following oral administration of eugenol or saline on Day 13 to 17, thermal and mechanical sensitivities were evaluated at 20 and 30 min postdose and values were averaged in each rat. Individual changes from baseline were calculated by subtracting the baseline value of thermal and mechanical sensitivity to the values measured over 5 days of treatment for each rat. Results of hyperalgesia and allodynia (raw measurements and change from baseline) following eugenol treatments were compared to those from the saline group every day using a two-sample *t*-test.

For the statistical comparison with the placebo group, homogeneity of variance in hyperalgesia and allodynia results was assumed considering the small number of animals used. Since comparisons between treatments were performed every day, the level of statistical significance was adjusted for the multiplicity of comparisons (Bonferroni correction). As a result, the level of significance was set to 0.01 (i.e., 0.05/5) considering that a total of 5 comparisons were performed. Statistical analyses were performed with SYSTAT, Version 8.2.

3. Results

3.1. Quantitative analysis

The full scan spectra of the dansyl chloride derivative of eugenol and the internal standard thymol displayed intense signals for the protonated molecular ion ($[M+H]^+$) at *m/z* of 398 and 384, respectively. As expected, the reactions resulted in a mass increase of 233 Da for each product. The mass transition in selected reaction monitoring mode was set for best sensitivity at 398 → 171 and 384 → 171 for eugenol and thymol, respectively. The protein precipitation approach was the simplest method available for preparation and resulted in a recovery >90%. A linear regression (weighted 1/concentration) was judged to produce the best fit for the concentration–detector relationship. The analytical range was set from 20 to 20000 ng/mL. The precision of the method was evaluated: the observed CV% range was from 7.1 to 12.1% and the observed accuracy range was from 89.1 to 107.8% of the nominal concentration.

Table 2
Mean (CV%) pharmacokinetic parameters of eugenol in plasma ($n=6$) and blood ($n=6$) following oral administration of eugenol (40 mg/kg) in male Sprague–Dawley rats

PK parameters	Plasma	Blood
AUC_{0-t} (μg h/mL)	0.384 (42.4%)	0.342 (31.0%)
AUC_{0-inf} (μg h/mL)	0.577 (51.0%)	0.518 (38.8%)
C_{max} (μg/mL)	0.123 (25.7%)	0.270 (80.5%)
T_{max} (h)[a]	2.13 (0.25–4.00)	0.25 (0.25–0.50)
$T_{1/2}$ (h)	14.0 (58.3%)	18.3 (37.3%)
CL/F (L/h/kg)	86.7 (50.0%)	86.8 (34.9%)

AUC_{0-t}: area under curve from time zero to the last measurable concentration, AUC_{0-inf}: area under curve extrapolated to infinity, C_{max}: maximum plasma concentration, T_{max}: time to maximum plasma concentration, $T_{1/2}$: terminal elimination half–life, CL/F: apparent oral clearance.

[a] Median (Minimum–Maximum).

Table 3
Partition coefficient (Kp) of eugenol in blood following a single gavage administration of eugenol (40 mg/kg)

Time (h)	Kp (RBC:Plasma)
0,25	1,8
0,5	2,8
1	0,4
2	0,5
4	0,9
6	0,6
8	0,1
24	0,2

3.2. Pharmacokinetic results

The pharmacokinetic profiles of eugenol in plasma and red blood cells were assessed in intact animals over 24 h. Mean concentration profiles are presented in Fig. 1. Overall, the mean plasma concentration profile of eugenol displayed double peaks at approximately 0.25 and 4 h and then declined very slowly over the 24 h kinetic study. Following an initial rapid decline, blood concentrations of eugenol also declined very slowly. Mean pharmacokinetic parameters of eugenol in plasma and blood are presented in Table 2. Mean AUC_{0-inf} values in blood and plasma were close to the AUC_{0-t}, suggesting that the extrapolated area of the curve was small relative to the calculated AUC_{0-inf}. Mean oral clearance values in plasma and blood were 86.7 and 86.8 L/h/kg, respectively. Mean $T_{1/2}$ values in plasma and blood were 14.0 and 18.3 h, respectively.

Partition coefficient (Kp) between RBC and plasma at all timepoints are presented in Table 3. Since a different group of animals was used for the determination of blood and plasma concentrations, the Kp was calculated using mean concentrations at each time point as an indication of the partition coefficient of eugenol *in vivo*. The RBC:plasma partition coefficient decreased over time, suggesting a re-distribution of eugenol from red blood cells to plasma. At early time points (15 and 30 min), the eugenol fraction in RBC is greater than plasma as suggested by its lipophilic properties.

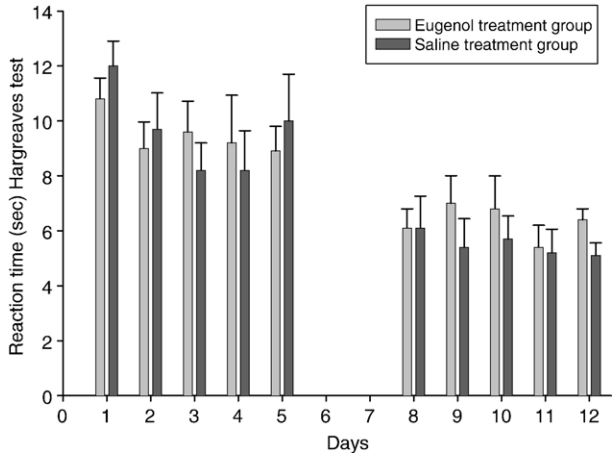

Fig. 2. Mean (±S.E.M.) thermal sensitivity (Hargreave's test) results during the behavioural period (Day 1–5) and the experimental phase of neuropathic pain (Day 8–12) in male Sprague–Dawley rats prior to eugenol ($n=6$) and saline treatments ($n=6$).

3.3. Pharmacodynamic results

Results of the Hargreave's test on the plantar heat sensitivity in male Sprague–Dawley rats before surgery (Day 1–5) and during evaluation of the experimental model of neuropathic pain (Day 8–12) are presented in Fig. 2. A statistically significant difference in plantar sensitivity was observed before and after the sciatic nerve cuff implantation ($P=0.03$) for the right paw thermal sensitivity testing.

Thermal sensitivity at baseline and following repeated oral administrations of eugenol and saline, as well as change from baseline values are depicted in Fig. 3. Overall, thermal sensitivity in rats receiving eugenol decreased constantly following repeated oral administrations of the drug. In sharp contrast to the eugenol group, thermal sensitivity in rats receiving the saline solution remained relatively stable over the 5 treatment days, with mean values ranging from 5.3 to 6.9 s. On the last day of treatment, mean (±S.E.M) reflex time of thermal sensitivity following eugenol and saline treatments were 11.4±1.23 and 6.1±0.53 s, respectively ($P<0.01$). Similarly, mean (±S.E.M.) change from baseline in thermal sensitivity following eugenol and saline treatments were +5.30±1.05 and +0.74±0.68 s, respectively

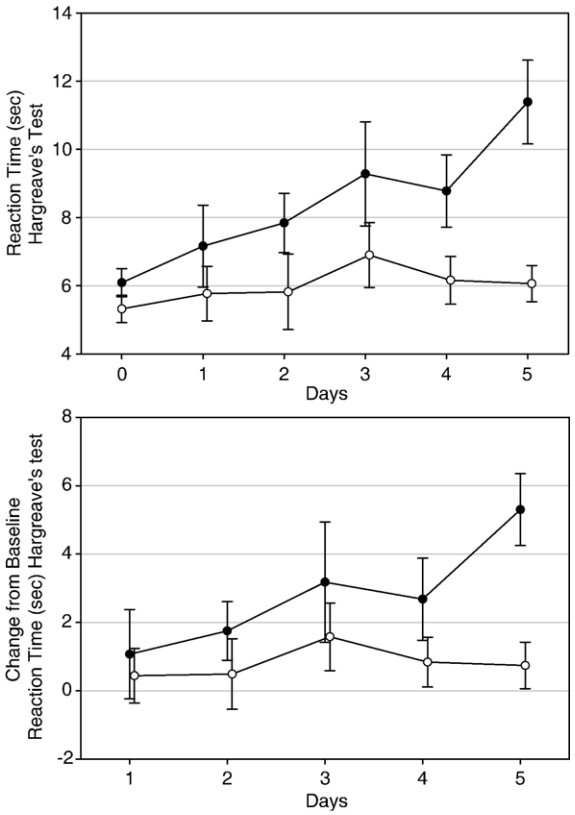

Fig. 3. Mean (±S.E.M.) thermal sensitivity results (Hargreave's test on cuff-implanted right hind paw) following repeated oral administrations of eugenol (40 mg/kg) (full circles) and saline (empty circles) in male Sprague–Dawley rats ($n=6$ per group). Day 0 corresponds to mean baseline values. On Day 5, $P<0.01$.

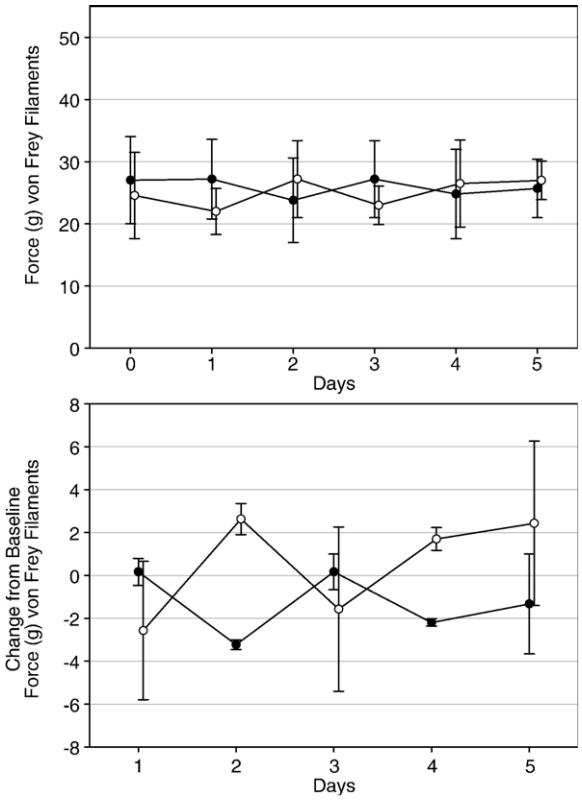

Fig. 4. Mean (±S.E.M.) mechanical sensitivity results (von Frey filaments test on cuff-implanted right hind paw) following repeated oral administrations of eugenol (40 mg/kg) (full circles) and saline (empty circles) in male Sprague–Dawley rats (n=6 per group). Day 0 corresponds to mean baseline values.

(P<0.01). Thermal sensitivity results are presented for the right hind paw (cuff-implanted) only, as no change was observed for the unoperated left hind paw (results not shown). Mechanical sensitivity at baseline and following repeated oral administrations of eugenol and saline, as well as change from baselines values are depicted in Fig. 4. Overall, mechanical sensitivity in rats receiving eugenol and saline treatments overlapped each other. No statistically significant differences were observed for right hind paws on any of the treatment days. Mechanical sensitivity results for the right hind paw were similar to those observed for the unoperated left hind paw (results not shown). No conclusion can be drawn for mechanical sensitivity since no allodynia was present in cuff-implanted animals and we have no explanation for this occurrence.

4. Discussion

The pharmacokinetics of eugenol was characterised in rats using a sensitive LC/MS/MS assay. Following the initial rapid decline in plasma concentrations of eugenol, a secondary peak occurred at approximately 4 h postdose. Since glucuronide and sulfate conjugates of eugenol were recently identified in urine of rats (Guenette et al., 2006), it is possible that the sudden increase in plasma concentration may be associated to enterohepatic recirculation of the drug such as previously reported for other phenolic compounds undergoing conjugation (Marier et al., 2002). Compartmental methods were not used in the current study since additional experimentations involving bile-cannulated rats would be required for the development of a pharmacokinetics model involving enterohepatic recirculation of eugenol. Mean elimination half-life of eugenol in plasma and blood were 14.0 and 18.3 h, respectively. These elimination half-life values of eugenol are markedly longer than those recently reported in a preliminary biodisposition study, where the metabolic fate of eugenol in plasma and urine was only characterised over a limited timeframe (Guenette et al., 2006). Due to the long elimination half-life of eugenol, some level of accumulation is expected following repeated oral administrations in rats.

The analgesic properties of eugenol were determined using the sciatic nerve cuff-implanted rat model inducing neuropathic pain (Pitcher et al., 1999). Results of the Hargreave's test confirmed the efficacy of the experimental model to induce neuropathic pain since sensitivity in male Sprague–Dawley rats was markedly reduced following sciatic nerve cuff implantation in the right hind paw of rats. Pain sensitivity was then assessed at baseline and following repeated oral administrations of 40 mg/kg of eugenol relative to a saline solution for 5 days. Although statistical significance between the eugenol and the saline group was only observed on Day 5, the pharmacological effect of eugenol appeared to increase in a constant manner over the 5 consecutive days of treatment. Thermal sensitivity results were reported for the right (cuff-implanted) hind paw only as no change from baseline was observed for the unoperated left hind paw (results not shown). Interestingly, these results suggest that eugenol did not affect the motor component of the reaction time in the Hargreave's test. No adverse clinical signs were observed in rats following oral administration of eugenol for 5 consecutive days.

These results suggest that eugenol may be used as a therapy for the treatment of neuropathic pain. Since eugenol appears to have a relatively long elimination half-life in plasma and red blood cells and since repeated daily oral administrations may result to a certain degree of accumulation of the drug, results of the current study suggest a long lasting blocking effect of eugenol on TRVP1 which resulted in a gradual decrease of pain sensitivity after 5 days of treatment. Other mechanisms may contribute to the alleviation of pain. Kanai et al. (2005) have shown that TRPV1 receptors may be up regulated in the sciatic nerve constriction model in rats. Eugenol may therefore show a greater effectiveness over time with a greater number of TRPV1 cell receptors in the development of neuropathic pain. This up regulation was observed in the dorsal horn of the lumbar spinal cord. We therefore cannot exclude that eugenol may modulate central as well as peripheral receptors (Ueda, 2006) in the development of neuropathic pain.

Eugenol is a reversible vanilloid agonist which is an antagonist for vanilloid receptors. Results from the current *in vivo* study are consistent with the previously published *in vitro* results showing that eugenol has capsaicine-like activity specific to the TRPV1 receptor, thereby confirming its potential role in neuropathic pain. Peripheral hypersensitised pain nerve endings and specific central nervous system loci including the dorsal horn of the spinal cord, the thalamus, the hypothalamus and the reticular formation (without any specific binding in the somatosensory cortex),

showed specific interactions with eugenol (Acs and Blumberg, 1994; Acs et al., 1996; Guo et al., 1999; Jancso-Gabor et al., 1970; Szallasi and Blumberg, 1993; Yang et al., 2003). Therefore other central targets apart from the dorsal horn of the spinal cord may be implicated in vanilloid mechanisms in neuropathic pain. In our study, the effects of eugenol on mechanical sensitivity were inconclusive since rats did not show allodynia. The effects of eugenol on this behavior need to be investigated since we have no evidence at the present time on mechanical desensitization with this drug. This will be the focus of our future studies. Other vanilloid receptors, such as TRPV4 (Suzuki et al., 2003), are implicated mechanical allodynia associated with neuropathic pain and there is no evidence at the present time to suggest binding of eugenol to other TRPV1 receptors. Other natural molecules may prove to be more potent than eugenol binding to other types.

Nociceptive information is relayed to spinal cord neurons of the dorsal horn, particularly in laminae I–II and V–VII (Todd et al., 2002). Lamina I neurons relay to different areas of the brain concerned with discrimination, affect, motor and autonomic regulations (Craig, 2002). These neurons receive inputs from Aδ and C nociceptive fibers responding to noxious thermal and mechanical stimulation (Bester et al., 2000; Lawson, 2002). The majority of small Δ and C nociceptive fibers are sensitive to vanilloid products and play a pivotal role in the control of pain sensitivity (Ikeda et al., 1997; Nichols et al., 1999). Capsaicin and resiniferatoxin are two non-reversible ligands of the vanilloid family used for the treatment of neuropathic pain (Szallasi and Blumberg, 1999). Autoradiography with [^{3}H] resiniferatoxin shows clear binding in laminae I and II of the dorsal horn of different mammalian species underlining the presence of RV in these anatomical levels (Szallasi et al., 1994). Also [^{3}H] resiniferatoxin interactions in dorsal root ganglions of rats induced currents that persist even after the removal of the initial agonist and therefore create a continuing effect on ganglion cells thus suggesting its potential for a prolonged and additive action (Baccaglini and Hogan, 1983; Forbes and Bevan, 1988; Heyman and Rang, 1985; Vlachova and Vyklicky, 1993; Winter et al., 1990). These results strongly suggest that eugenol may also act by inducing a block in pain transmission.

Overall, our results support the hypothesis that eugenol may alleviate neuropathic pain. Only the cuff model in rats was used for these experiments and to establish its efficacy eugenol should be tested using other neuropathic pain models related sensitization by TRPV1 receptors. Further work is also necessary to evaluate the dose effect relationship of eugenol as well as central and peripheral contributions.

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