RESEARCH PAPER

Preliminary pharmacokinetics of tramadol hydrochloride after administration via different routes in male and female B6 mice

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Abstract

Objective 1) To determine the pharmacokinetics of tramadol hydrochloride and its active metabolite, *O*-desmethyltramadol (M1), after administration through different routes in female and male C57Bl/6 mice; 2) to evaluate the stability of tramadol solutions; and 3) to identify a suitable dose regimen for prospective clinical analgesia in B6 mice.

Study design Prospective, randomized, blinded, parallel design.

Animals A total of 18 male and 18 female C57Bl/6 mice (20–30 g).

Methods Mice were administered 25 mg kg^{-1} tramadol as a bolus [intravenously (IV), intraperitoneally (IP), subcutaneously (SQ), orally per gavage (OS_{gavage})] over 25 hours [orally in drinking water (OS_{water}) or Syrspend SF (OS_{Syrsp})]. Venous blood was sampled at six predetermined time points over 4 to 31 hours, depending on administration route, to determine tramadol and M1 plasma concentrations (liquid chromatography and tandem mass spectrometry detection). Pharmacokinetic parameters were described using a noncompartmental model. The stability of tramadol in water (acidified and untreated) and Syrspend SF (0.20 mg mL^{-1}) at ambient conditions for 1 week was evaluated.

Results After all administration routes, C_{max} was >100 ng mL^{-1} for tramadol and >40 ng mL^{-1} for M1 (reported analgesic ranges in man) followed by short half-lives (2–6 hours). The mean tramadol plasma concentration after self-administration remained >100 ng mL^{-1} throughout consumption time. M1 was found in the OS_{Syrs} group only at 7 hours, whereas it was detectable in OS_{water} throughout administration. Tramadol had low oral bioavailability (26%). Short-lasting side effects were observed only after IV administration. Water and Syrspend SF solutions were stable for 1 week.

Conclusions and clinical relevance 1) At the dose administered, high plasma concentrations of tramadol and M1 were obtained, with half-life depending on the administration route. 2) Plasma levels were stable over self-consumption time. 3) Solutions were stable for 1 week at ambient conditions.

Keywords analgesia, mice, pharmacokinetics, tramadol.

Introduction

Mice are widely used as laboratory models for surgical procedures. The provision of appropriate analgesia for peri- and postoperative pain is an ethical and legal imperative (Carbone 2011) and essential for scientific integrity as untreated pain is expected to

affect the outcome data. However, providing an effective analgesic treatment for the target species is challenging for involved scientists (i.e. veterinarians, researchers, animal welfare bodies) because of the biological peculiarities, the sparse published data of both the pharmacokinetics and efficacy of potentially relevant analgesics in the target species or strain, and finally the potential for interaction with the experimental readout.

Mice as a prey species tend to hide signs of pain, which hampers the recognition and quantification of pain, contributing to the underuse of postoperative analgesics. To date, the spectrum of analgesics available for laboratory mice relies mainly on few opioids (i.e. buprenorphine) and nonsteroidal anti-inflammatory drugs (NSAIDs; carprofen, meloxicam). While offering potentially good analgesic options for mice (Tubbs et al. 2011; Oyama et al. 2012; Jirkof et al. 2015), they have limitations. NSAIDs are accompanied by anti-inflammatory and immune-modulatory effects (Iñiguez et al. 1999; Paccani et al. 2002); hence, are inappropriate for studies involving inflammation and the immune system. Additionally, their efficacy is questionable based on the latest evidence (Roughan et al. 2016).

The μ-agonist opioids, apart from interfering with the immune response to some extent (Page 2005; Franchi et al. 2007; Ricardo Buenaventura et al. 2008), present dose-dependent undesirable side effects, such as respiratory and gastrointestinal depression, tolerance, hyperalgesia or increased activity (Flecknell 1984; Hayes et al. 2000; Hau & Schapiro 2002; Ricardo Buenaventura et al. 2008; Grimm et al. 2015).

As a result of its relatively high benefit/risk ratio, favourable pharmacokinetic (PK) properties, low potential for drug interactions in humans and other animal species (Lewis & Han 1997), and noncontrolled substance schedule, tramadol might be an interesting candidate to widen the analgesic portfolio in mice. Actual evidence of the analgesic efficacy of tramadol in mice is controversial: a recent study demonstrated that tramadol ameliorates cyclophosphamide-induced bladder-pain-related behaviours in mice (3–10 mg kg^{-1}, orally) (Oyama et al. 2012), whereas Wolfe et al. (2015) do not recommend it as a sole analgesic after abdominal laparotomy in mice. However these studies had no PK profiles supporting the dynamic data.

Pain treatment can be further improved by optimizing the methods of administration, long-lasting methods with the least stress possible (i.e. sustained/controlled release formulations or self-administration methods). Self-administration methods are an attractive option because they could ensure stable drug levels in the blood, which is necessary for adequate (in both intensity as duration) analgesic coverage while avoiding repetitive handling of the animals, thereby minimizing the stress. Therefore, in recent years different types of vehicles (water, pellets, hazelnut cocoa spread, jelly) have been tested for voluntary oral drug delivery in laboratory animals with some success. There are already available data supporting this route of administration for analgesics (i.e. buprenorphine) in rats and mice (Abelson et al. 2012; Molina-Cimadevila et al. 2014); however, to the best of our knowledge, there is no published data regarding tramadol delivery in mice by such means.

Based on the rationales expressed and knowledge gaps in the literature, this study aims to: 1) determine the pharmacokinetics of tramadol and *O*-desmethyltramadol (M1) after tramadol administration through different routes in female and male B6 mice; 2) evaluate the stability of tramadol in aqueous solution, to explore the feasibility of using drinking water for tramadol delivery; and 3) determine the most suitable route or combination of routes for this strain.

Materials and methods

Animals

The experimental protocol was approved by the local veterinary authorities (Kanton BS, Switzerland, TVB # 2768). Based on the planned PK profile design, 18 male and 18 female C57BL/6J mice [20–30 g body weight (BW)] were used, the rationale behind this number of mice was to use the minimum amount of mice necessary that allowed performing the analysis. Inclusion criteria were: strain, age and BW, healthy on clinical examination and based on review of health reports (according to Federation of European Laboratory Animal Science Associations (FELASA) health monitoring recommendations) (Mähler et al. 2014). Exclusion criteria were as follows: failure to adhere to pretest requirements or overt sign of illness.

Mice were housed in groups of three animals in standard polycarbonate cages, with aspen wood bedding (J. Rettenmeier & Söhne GmbH, Germany) and nesting material. A rotational enrichment plan was in place, with hemp rope (Cordag AG, Switzerland) and aspen wood stick (LAB & VET

Service GmbH, Austria) present in the home cages. Mice were acclimated in a reverse 12-hour light−dark cycle (lights on at 6 PM and off at 6 AM) for a minimum of 7 days before the start of the study and kept in rooms with controlled temperature (20−22 °C) and relative humidity (40−60%). Animals were housed in the same room in which the study was performed and had free access to a rodent maintenance diet (Mouse and Rat Maintenance 3436; Kliba Nafag AG, Switzerland) and tap water. The facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi).

Study design

In vitro

The stability of tramadol when diluted in different types of drinking water and Syrspend SF PH4 Aroma-free (Fagron, Germany) was evaluated by a method similar to that described by Ingrao et al. (2013). Tramadol hydrochloride (0.6 mL, Tramal 100 injection solution; Grünenthal Group, Germany) was added to 150 mL water [bottle 1: nonacidified water, F. Hoffmann-La Roche (IWB Basel-Stadt, Switzerland); bottle 2: nonacidified water, Unispital Zürich (EWZ Zürich, Switzerland); bottle 3: acidified water, F. Hoffmann-La Roche; bottle 4: acidified water, Unispital Zürich; or bottle 5: Syrspend SF] to achieve a final tramadol concentration of 0.20 mg mL^{-1}. The acidified water was first filtered and demineralized and then acidified by adding hydrogen chloride (ProMinent Dosiertechnik AG, Switzerland) until a pH of 2.6−3.0 was reached (equipment for filtration, deminaralization and acidification provided by ProMinent Dosiertechnik AG).

All bottles were stored at ambient light and temperature conditions for up to 1 week. Samples were taken with a syringe into 2-mL Eppendorf tubes (VWR International, Switzerland) at 0, 24, 48 and 72 hours and 7 days after the preparation of the solution and stored at −20 °C until analysis (Musshoff et al. 2006; Cooper & Negrusz 2013).

In vivo

Animals were assigned randomly using a prospective, blinded, parallel design (three females and three males per group) to one of six administration groups: 1) intravenous (IV); 2) intraperitoneal (IP); 3) subcutaneous (SQ); 4) oral per gavage (OS_{gavage}) as a single dose; 5) oral in drinking water (OS_{water}) for 25 hours; and 6) oral in Syrspend SF (OS_{Syrsp}) for 25 hours.

All groups were administered 25 mg kg^{-1} of tramadol hydrochloride. Because of the small volume required, a dilution of Tramal 100 in NaCl 0.9% (B. Braun Medical AG, Switzerland) with a final concentration of 6.25 mg mL^{-1} was used for the IV, IP, SQ and OS_{gavage} administration groups. A solution of 0.20 mg mL^{-1} was prepared as described above for the OS_{water} and OS_{Syrsp} groups and administered in water bottles with sipper. Assuming an average daily water intake of 3.75 mL and 25 g BW, a solution of 0.20 mg mL^{-1} would ensure a dose of 25 mg kg^{-1} tramadol in 24 hours (Harkness et al. 2013; Ingrao et al. 2013).

On the morning of the experiment, the cage mates ($n = 3$) were placed in metabolic cages. The person administering the tramadol then randomly assigned each individual to one of the six treatments by drawing lots. A second operator, who was unaware of the administration routes, performed the blood collection at the determined time points after drug administration. Blood (volume, 50 µL) was drawn twice from each animal by tail-vein puncture with the conscious mice placed in a restrainer. The detail of the experimental setup is depicted in Fig. 1.

All samples were collected into lithium−heparin-wetted tubes (POCT 50 µL LH Minnivette; Sarstedt AG & Co, Germany) and stored on dry ice; plasma was separated by centrifugation at 3000 ***g*** for 10 minutes and frozen at −20 °C until analysis (Musshoff et al. 2006; Cooper & Negrusz 2013).

Liquid chromatography and tandem mass spectrometry

Tramadol and M1 plasma concentrations were determined using liquid chromatography (LC) (Shimadzu Prominence, MD, USA) coupled with mass spectrometry API 5500 (MS/MS) (ABSciex, MA, USA). Plasma samples were processed using a protein precipitation procedure. Methanol (volume, 30 µL) was added to 0.5 µL of plasma in a 384-well plate, agitated and centrifuged at 3512 g for 10 minutes. Then 3 µL supernatant was injected into the liquid chromatography and tandem mass spectrometry (LC-MS/MS).

The mobile phase consisted of: A) 10 mM ammonium acetate in water + 0.05% acetic acid (v/v) and B) methanol + 0.05% acetic acid (v/v). The flow rate was 0.8 mL $minute^{-1}$. The run gradient started at 95% A/5% B for 0.3 minutes then with a linear

a)

Mouse #		Sampling times post SQ/Oral (gavage) dosing					
♀	♂	15 minutes	30 minutes	1 hour	2 hours	4 hours	7 hours
1	4	x			x		
2	5		x			x	
3	6			x			x

b)

Mouse #		Sampling times post IP/IV dosing					
♀	♂	5 minutes	15 minutes	30 minutes	1 hour	2 hours	4 hours
1	4	x			x		
2	5		x			x	
3	6			x			x

c)

Mouse #		Sampling times from start of drinking water /Syrspend SF self-dosing					
♀	♂	1 hour	3 hours	7 hours	25 hours*	26 hours	31 hours
1	4	x			x		
2	5		x			x	
3	6			x			x

Figure 1 Blood sampling setup: three mice per sex were allocated to each administration group and two 50-μL samples per mouse were collected. These data points were used to create a composite pharmacokinetic profile.* replaced with plain solution; #, number; ♀, female mice; ♂, male mice; IP, intraperitoneal; IV, intravenous; SQ, subcutaneous.

gradient to 20% A/80% B over 0.6 minutes, followed by a linear gradient to 5%/95% B over 1.2 minutes, returning to 95% A/5% B over 1.8 minutes with a linear gradient. The total run time was 3.9 minutes. Separation was achieved using a 2.1 × 30.0 mm column (C18 Ascentis Express; Supelco; Sigma-Aldrich Corporation, Switzerland) maintained at 50 °C.

The transition was parent ion (*m*/*z*) 263.9, daughter ion (*m*/*z*) 58.1 for tramadol and parent ion (*m*/*z*) 249.9, daughter ion (*m*/*z*) 58.1 for M1. The analytical range was 5−10,000 ng mL^{-1} for tramadol and 25−10,000 ng mL^{-1} for M1. Standards extracted from spiked blank plasma gave calibration curves over the dynamic range. The quality control (QC) samples were run in replicates of two at a concentration of 5, 50 and 500 ng mL^{-1} for tramadol. The accuracy was assessed at each standard and QC level, and all data points were within ±15% or ±20% at the lower limit of quantification (ULOQ) and upper limit of quantification (LLOQ), respectively.

Pharmacokinetic and data analysis

Noncompartmental PK analyses (NCA) were performed using a computer software (Phoenix WinNonlin Version 6.4; Pharsight Corp., CA, USA). The following PK parameters were calculated from the composite plasma concentration data after IV administration: the area under the curve (AUC_{last}) from time 0 to the last time point above the analytical LLOQ, the AUC extrapolated to infinity (AUC_{inf}), the percentage of the AUC_{last} extrapolated to infinity (AUC_{extrap}), plasma clearance (Cl), terminal half-life ($t_{1/2}$), terminal rate constant (λz), volume of distribution at steady state (Vd) and apparent volume of distribution of the area during the elimination phase (Vz). The concentration at time 0 (C_0) was calculated by log linear back extrapolation using the first two time points after IV administration. The λz was determined using at least three time points. PK parameters after extravascular administration (IP, SC, OS_{gavage}) from the composite plasma concentration data included the AUC_{last},

AUC_{inf}, $t_{1/2}$, λz, Cl per fraction of the dose absorbed (F), and apparent volume of distribution of the area during the elimination phase per fraction of the dose absorbed (Vz/*F*). The *F* after extravascular administration was determined by dividing the oral AUC_{inf} by the IV AUC_{inf}. The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were determined directly from the plasma concentration data. The ratios of the tramadol and M1 AUC_{last} after different routes of administration were calculated where data permitted.

Results

In vitro

The measured concentration of tramadol dilution in water (both acidified and nonacidified) and Syrspend SF ranged from 0.193 to 0.230 mg mL^{-1} (time 0 in Syrspend SF was considered an artefact and therefore excluded), and remained stable for 7 days under ambient conditions (Fig. 2).

The measured concentration of the injectable tramadol diluted in NaCl 0.9% (targeted to achieve a final concentration of 6.25 mg mL^{-1}) used for the parenteral and OS_{gavage} administration ranged between 6.24 and 6.34 mg mL^{-1}, confirming the accuracy of the extemporaneous solution and was stable for 24 hours under ambient conditions.

In vivo

After IV administration, mild to moderate incoordination, ataxia and *straub* tail were observed in all animals, the duration of these side effects being no longer than 40 seconds. In all other treatment groups, no clinically evident side effect was observed.

Individual and mean plasma concentrations of tramadol and M1 were plotted *versus* time for each administration route (Figs. 3 & 4). The PK parameters for tramadol and M1 calculated from the composite concentration data for both sexes combined are presented in Tables 1 and 2.

Following IV administration, tramadol and M1 were quantifiable up to 4 and 2 hours after administration, respectively. Tramadol had high systemic clearance (approximately 160 mL $minute^{-1}$ kg^{-1}), a large volume of distribution (Vd >7.2 L kg^{-1}) and a short plasma half-life (<1 hour). C_{max} and AUC_{last} values for M1 were 15% and 20% of the respective values for tramadol.

Following IP administration, tramadol and M1 were quantifiable up to 4 and 2 hours after administration, respectively. The bioavailability of tramadol was 67%.

Following SQ administration, tramadol and M1 were quantifiable up to 7 hours after administration. AUC_{last} values for tramadol were comparable between the two routes. Furthermore, the estimated AUC_{last} for M1 after SQ administration was twofold or greater higher than M1 exposure after IV dosing, likely as a result from the prolonged absorption of the parent drug.

Following OS_{gavage} administration, tramadol and M1 were quantifiable up to 7 hours after administration. The peak plasma concentration was reached 1 hour after administration. The oral bioavailability was low (26%) and reflected the extent of absorption and presystemic clearance because of metabolism.

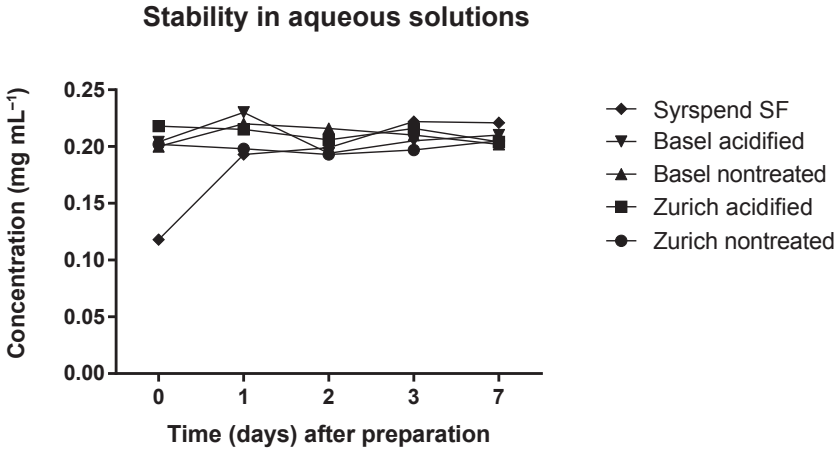

Figure 2 Stability of tramadol HCl injectable solution (Tramal 100, 50 mg mL^{-1}) in Syrspend SF, Basel acidified, Basel nontreated, Zurich acidified and Zurich nontreated water and held at ambient conditions for 7 days.

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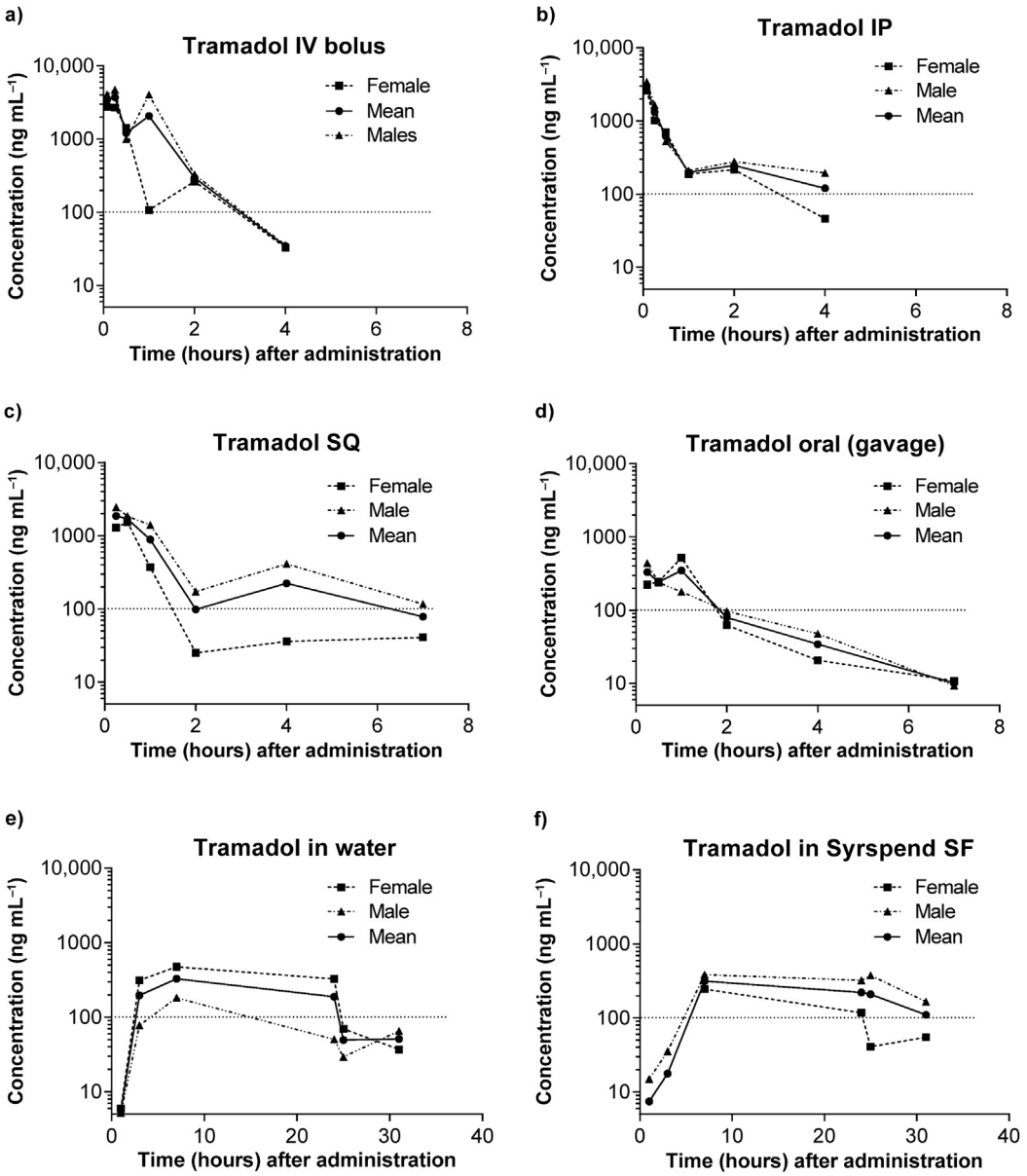

Figure 3 Semi-log composite plots of female, male and mean plasma tramadol concentrations over time after a single dose of 25 mg kg^{-1} tramadol via a) intravenous (IV), b) intraperitoneal (IP), c) subcutaneous (SQ) and d) oral (gavage) administration routes. Semi-log composite plots of female, male and mean plasma tramadol concentrations of a dose of 25 mg kg^{-1} tramadol administered voluntarily during 25 hours in e) water and f) Syrspend SF. At 25 hours the bottles containing tramadol and water/Syrspend SF were replaced by bottles with plain solutions. The horizontal line represents the minimal effective analgesic plasma level of tramadol (100 ng mL^{-1}) in humans.

The apparent $t_{1/2}$ for the extravascular routes (1.4 hours IP and 1.7 hours OS_{gavage} and SQ) was twice the value of the estimated half-life after IV dosing and is suggestive of 'flip-flop' kinetics. Flip-flop occurs when the rate of absorption is slower than the rate of elimination, and here the absorption was the rate-limiting step in tramadol systemic disposition and elimination. Accordingly, the estimated half-lives represent the absorption half-lives. The concentration profiles after IP and SC administration (Figs. 3b & c) are suggestive of biphasic absorption and/or enterohepatic recycling, but no definitive conclusion could be made because of the limited number of animals and sampling time points.

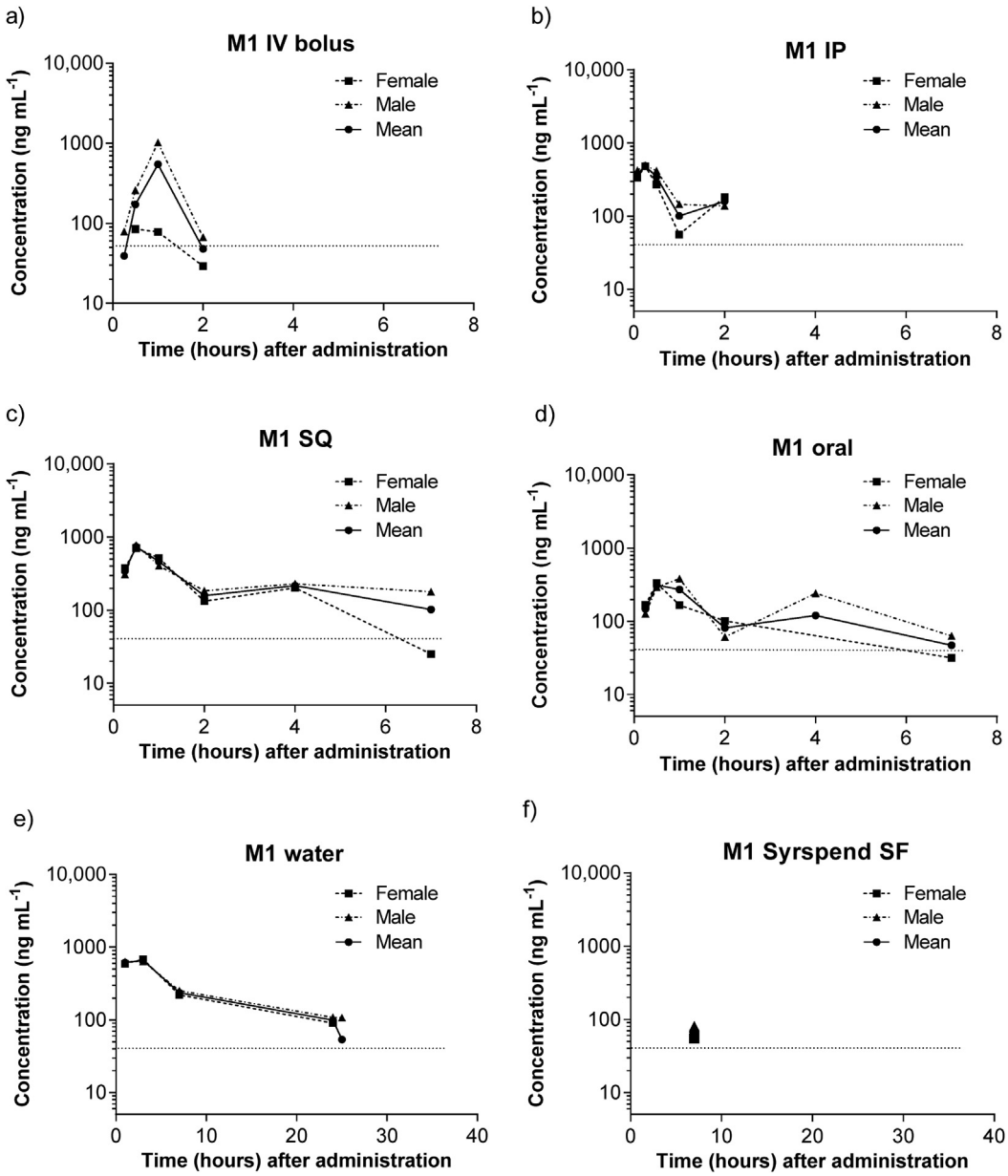

Figure 4 Semi-log composite plots of female, male and mean plasma *O*-desmethyltramadol (M1) concentrations over time after single dose of 25 mg kg^{-1} tramadol via a) intravenous (IV), b) intraperitoneal (IP), c) subcutaneous (SQ) and d) oral (gavage) administration routes. Semi-log composite plots of female, male and mean plasma tramadol concentrations of a dose of 25 mg kg^{-1} tramadol administered voluntarily during 25 hours in e) water and f) Syrspend SF. At 25 hours, the bottles containing tramadol and water/Syrspend SF were replaced by bottles with plain solutions. The horizontal line represents the minimal effective analgesic plasma level of M1 (40 ng mL^{-1}) in humans.

The minimal tramadol plasma concentration for analgesic effect in humans has been estimated to be 100 ng mL^{-1} (Lewis & Han 1997); however, the threshold concentration in mice is not known. We used the 100 ng mL^{-1} concentration as a target level to achieve and maintain when comparing the dosing routes. Tramadol concentrations $\geq$100 ng mL^{-1} were maintained over 2 hours after IV and for up to 4 hours after IP or OS_{gavage} administration, whereas after SQ administration, values remained >100 ng mL^{-1} for almost 6 hours.

After OS_{Syrsp} and OS_{water} administration, C_{max} values were similar (328 and 315 ng mL^{-1}, respectively). Almost 6 hours were necessary for the OS_{Syrsp} group to

Table 1 Pharmacokinetic parameters for tramadol and M1 in B6 mice. A single dose of 25 mg kg^{-1} of tramadol was administered via intravenous (IV), subcutaneous (SQ), intraperitoneal (IP) and oral gavage routes. Data are reported as mean ± standard error

			Dosing route			
Parameter	**Units**	**Analyte**	**IV**	**Oral**	**IP**	**SQ**
C_0	ng mL^{-1}	Tramadol	3450	NA	NA	NA
		M1	NA	NA	NA	NA
C_{max}	ng mL^{-1}	Tramadol	3710 ± 1010	347 ± 170	3010 ± 410	1870 ± 575
		M1	549 ± 272	313 ± 12	487 ± 5	735 ± 20
T_{max}	hours	Tramadol	0.25	1	0.08	0.25
		M1	1	0.5	0.25	0.5
T_{last}	hours	Tramadol	4	7	4	7
		M1	2	7	2	7
AUC_{last}*	hours ng^{-1} mL^{-1}	Tramadol	2580 ± 265	652 ± 43	1520 ± 213	2760 ± 1060
		M1	509 ± 233	850 ± 208	434 ± 33	1630 ± 86
AUC_{inf}*	hours ng^{-1} mL^{-1}	Tramadol	2610	677	1750	2940
		M1	NC	1010	NC	2040
AUC_{extrap}	%	Tramadol	1.4	3.6	13.3	6.4
		M1	NC	15.9	NC	19.9
Cl or Cl/*F*	mL $hour^{-1}$ kg^{-1}	Tramadol	159	616	238	141
Vd	mL kg^{-1}	Tramadol	7200	NA	NA	NA
Vz/*F*	mL kg^{-1}	Tramadol	10,100	89,400	27,700	20,400
$t_{1/2}$	hours	Tramadol	0.73	1.68	NR	1.67
		M1	NC	2.37	1.17	2.76
F		Tramadol	NA	0.26	0.67	1.12
		M1				
M1/Tramadol ratio (C_{max})		Tramadol	NA	0.26	0.67	1.12
		M1	0.20	1.30	0.29	0.59

AUC_{extrap}, percent of the AUC extrapolated; AUC_{inf}, area under plasma concentration-time curve from time 0 to infinity; AUC_{last}, area under the concentration curve from time zero to the last quantifiable time point; B6, C57Bl/6; Cl, plasma clearance; Cl/*F*, plasma clearance per fraction of the dose absorbed; C_0, concentration at time 0; C_{max}, maximum plasma concentration; *F*, fraction of the dose absorbed; M1, *O*-desmethyltramadol; NA, not applicable; NC, not calculable; NR, not reportable ($r^2 < 0.8$); T_{max}, time to reach C_{max}; T_{last}, the time of the last measurable concentration; T_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life; Vd, volume of distribution at steady state; Vz/*F*, apparent volume of distribution of the area during the elimination phase per fraction of the dose absorbed.
*Water/Syrspend SF with tramadol were replaced with plain solution.

Table 2 Pharmacokinetic parameters (mean ± standard error) for tramadol and M1 in mice after self-administration in drinking water (from composite data of both sexes combined). The target dose was 25 mg kg^{-1} 24 $hours^{-1}$ based on water consumption of 3 mL $animal^{-1}$ 24 $hours^{-1}$. M1 was quantifiable at a single time point for both sexes and, therefore, no pharmacokinetic parameters were calculated reported

Analyte	**Formulation**	**C_{max} (± SE) (ng mL^{-1})**	**$AUC_{(0-25h)}$ (hours* ng^{-1} mL^{-1})**	**AUC_{last} (±SE) (hours* ng^{-1} mL^{-1})**
Tramadol	T in Syrspend SF	315 ± 70	5450	6170 ± 2240
	T in water	328 ± 146	4330	4630 ± 1940
M1	T in water	658 ± 20	6280	6280 ± 239

AUC_{0-25h}, area under the concentration curve from time 0 to 25 hours; AUC_{last}, area under the concentration curve from time 0 to the last quantifiable time point; C_{max}, maximum plasma concentration; M1, *O*-desmethyltramadol; SE, standard error; T, tramadol; T_{max}, time to reach C_{max}.
*Water/Syrspend SF with tramadol were replaced with plain solution.

reach mean tramadol plasma levels >100 ng mL^{-1}, but it remained stable (>200 ng mL^{-1}) until replacement of the drinking bottle with plain drinking water. Conversely, mean tramadol concentrations >150 ng mL^{-1} were reached after only 3 hours in the OS_{water} group and remained >100 ng mL^{-1} during consumption time (Figs. 3e–f).

High M1 concentrations (>100 ng mL^{-1}) were found in the OS_{water} group during consumption time, but plasma concentration halved at 26 hours, 1 hour

after stopping the treatment. In the OS_{Syrsp} group, M1 could be detected only at the 7-hour time point (67.5 ng mL^{-1}) (Figs. 4e–f).

Unfortunately, as a result of unexpected leaks in several bottles, it was not possible to quantify the water and Syrspend SF intake as planned.

Discussion

The disposition and pharmacokinetics of a potentially clinically relevant dose of tramadol and its metabolite M1 after different routes of administration have been characterized in female and male B6 mice.

The plasma levels of both tramadol and M1 in B6 mice were high and in the analgesic range described for humans (Lehmann et al. 1990; Lewis & Han 1997) for up to 2 hours after IV administration with the greatest duration (6 hours) after SQ administration. Therefore, the expected analgesic effect of a single dose of tramadol in this strain is likely to be of short duration depending on the administration route. Oral bioavailability after a single gavage dose was lower than that reported in other species (Lintz et al. 1986; Pypendop & Ilkiw 2008). However, when tramadol was administered in water or Syrspend SF, the plasma levels achieved were high and remained stable during the consumption time (25 hours), which implies that animals kept voluntarily drinking the solution during that time, including their inactive phase during the day.

In compliance with the 3R principles (Russell et al. 1959; Carbone 2011), a sparse sampling design was used in this study with the minimal number of animals ($n = 2$ time points per sex). The data did not allow PK analysis beyond NCA; however, the results of the analysis are sufficient for assessing the exposure of tramadol in mice after different administration routes and prospective dose range finding and efficacy studies.

The dose tested in the present study was selected based on information obtained from an extensive literature review and the extrapolation of doses from other species, especially rats where doses range from 4 to 50 mg kg^{-1} after oral, IP or SC administration (Cannon et al. 2010). Referenced doses for mice are sparse, and the dose of tramadol in drinking water recommended by the Board for Anesthesia and Analgesia of the GV-SOLAS (Henke et al. 2015) for use in mice is 125 mg kg^{-1} of tramadol in 24 hours, which is up to five times higher than the dose used in the current study. However, no PK/pharmacodynamic (PD) data supports the use of such a high dose. High doses increase the likelihood of side effects and bias research outcomes, which are highly undesirable when using analgesics in experimental mice studies (Gaspani et al. 2002). Therefore, in the current study a more conservative dose was selected, and overall, the PK parameters of tramadol reported in the current study are in accordance with those obtained by Matthiesen et al. (1998) after administration of 30 mg kg^{-1} (orally) in mice. In both studies, high plasma concentrations (C_{max} <300 ng mL^{-1}), short half-life (<2 hours) and similar AUC values were obtained. No comparisons can be made for the other administration routes.

Tramadol-induced analgesia results from both a monoaminergic and an opioidergic effect. The monoaminergic effect is activated mostly by tramadol, whereas M1, which is up to six times more potent as analgesic as tramadol, is mainly responsible for the opioidergic effect. Therefore, the metabolism of tramadol is key to its analgesic action. Tramadol is metabolized in the liver by the cytochrome p450 enzyme system and is responsible for the variability among species in the pharmacokinetics and metabolism (Raffa et al. 1993; Wu et al. 2001; Martignoni et al. 2006) and consequent analgesic efficacy of tramadol (Desmeules et al. 1996; Saleem et al. 2014). In the current study, M1 concentrations paralleled tramadol concentrations after all administration routes, suggesting a rapid metabolism of tramadol to M1 in B6 mice. The faster metabolism in rodents compared to other species (Matthiesen et al. 1998; White & Seymour 2005) could explain the low bioavailability (F) found in mice in the current study (26%) after a single oral dose compared to humans (70%; Lintz et al. 1986), horses (64%; immediate release capsules; Giorgi et al. 2007) or cats (93%; Pypendop & Ilkiw 2008). Nonetheless, a species-specific lower absorption of tramadol in the small intestine could contribute to a low bioavailability. Results also show a rather short half-life for both tramadol and M1 for mice in any of the routes tested compared to humans (Lewis & Han 1997). As a result of the short elimination half-life observed, a frequent dosing will be required to maintain targeted plasma concentrations.

In humans, the reported minimal effective analgesic plasma concentration (MEC) is 100 ng mL^{-1} for tramadol and 39.6 ± 29.5 ng mL^{-1} for M1 (Lehmann et al. 1990; Lewis & Han 1997). In the present study, mean tramadol C_{max} values were >300 ng mL^{-1} for all routes of administration, and plasma concentrations of both tramadol and M1 were above the aforementioned minimal effective analgesic

concentration range for humans. Hence, if the MECs reported in humans apply to mice, it might be expected to achieve some level of analgesic effect with the dosage selected (25 mg kg^{-1}). The duration of the analgesic effect would vary according to the route; however, this needs to be assessed in an appropriate PK/PD experimental setting.

In this study, although the highest plasma concentrations of both tramadol and M1 in mice were observed in the IV and IP groups, their concentrations decreased very fast. The M1 concentrations were below the LLOQ after 2 hours of the treatment; hence, no analgesic effect can be expected after this time point.

The most clinically relevant PK profile was achieved after SQ administration as the serum concentrations of both tramadol and M1 exceeded the MEC for humans (T = 100 ng mL^{-1} and M1 = 40 ng mL^{-1}) and remained above these concentrations for as long as 6 hours. Therefore, tramadol administered SQ could provide an early onset of and continuous antinociception for up to 6 hours in B6 mice.

The half-life of tramadol and M1 in mice after a single dose was short, independent of the administration route, meaning that repetitive injections become necessary to achieve constant effective plasma levels over time. In laboratory rodents, handling is associated with stress (Sharp et al. 2002; Jirkof et al. 2015) and should be avoided, not only for animal welfare but also because stress can bias the outcome data. Thus, self-administration methods or sustained release formulations are very attractive because they may ensure stable blood drug levels while avoiding repetitive handling of the animals.

Before use, the stability of tramadol in both vehicles (drinking water and Syrspend-SF) for up to a week was confirmed, warranting its practical use under the same conditions that we find in our animal facility. The time to reach C_{max} with auto-consumption was 3 and 7 hours for the mice in OS_{water} and OS_{syrsp}, respectively. Mice are a neophobic species (Kronenberger & Médioni 1985; Molina-Cimadevila et al. 2014), and the lack of habituation to Syrspend SF before the beginning of the study might have contributed to this outcome. Nevertheless, a lower intestinal absorption rate of tramadol in Syrspend SF could be of importance as well.

Plasma levels were high and constant over consumption time in both self-administration groups, proving that tramadol in water and Syrspend SF and at the dose chosen (25 mg kg^{-1} in 24 hours) is palatable and well accepted by the animals. An interesting finding was that M1 was only detectable at the 7-hour time point in group OS_{Syrsp}. After ruling out any analytical issues and a possible interference of any of the components of Syrspend SF in the metabolism of tramadol, this observation remains currently unexplained. It is possible that Syrspend SF, a food starch-based vehicle, slowed the rate of intestinal absorption of tramadol. Thus, as tramadol was absorbed, it was transformed to M1 and eliminated without reaching measurable concentrations in the plasma. This could account also for the delayed T_{max} observed in this group, because tramadol does not accumulate in blood until hepatic saturation.

In the present study, several mild and very transient side effects (incoordination and *straub* tail) (Zarrindast et al. 2001) were observed after IV administration. These observations are in accordance with the seizures and sedation reported in mice and rats (Osterloch et al. 1978; Raffa & Stone 2008; Cannon et al. 2010), and dizziness, headache, nausea and dry mouth reported in humans (Lewis & Han 1997). Slow IV injection has been recommended to avoid side effects in humans and other species (Lewis & Han 1997; McMillan et al. 2008; Shilo et al. 2008); however, in mice, this would be difficult to implement because of the small required volume and prolonged handling stress. In rats, skin lesions following SQ administration (25 mg kg^{-1}) have been reported (Cannon et al. 2010); however, this was not observed in any of the B6 mice.

In this study, females appeared to have consistently lower concentrations of tramadol than males, except for the OS_{water} group. A relevance of possible sex differences needs to be established in future studies with a larger sample size. In addition, B6 mice were chosen because this is the most widely used strain in research; however, further studies for the evaluation of possible strain differences would be of interest.

This study provides basic PK parameters for tramadol in B6 mice: 1) at a 25 mg kg^{-1} dose, high plasma concentrations of both tramadol and M1 were obtained followed by a short half-life of 2 to 6 hours depending on the administration route; 2) tramadol was stable in aqueous solutions for up to 1 week at ambient conditions and 3) self-administration of medicated water containing tramadol at a concentration of 0.20 mg mL^{-1} was successful in achieving constant plasma levels over consumption time.

The current study provides data for selecting clinically relevant dose regimens for further pharmacodynamic testing in order to quantify objectively antinociceptive activity of tramadol in this mouse strain.

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Author contributions

REV, AB and DID: design, analysis and interpretation of data and drafting of the manuscript. REV and CR: *in vivo* work. FA: LC-MS/MS analysis.

Conflict of interest statement

Authors declare no conflict of interest.

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Appendix A. Supplementary data

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