Juliane Rudeck, 2019-04-02
DOI 10.17590/asr.0000091

Exemplary study for Animal Study Registry “Dose-response curve for buprenorphine in mice”

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Summary

Preamble: This study serves as an exemplary exploratory study for Animal Study Registry which was already carried out in 2016.

Opioids are the treatment of choice for moderate to severe perioperative pain in mice. Among the numerous opioid drugs available, buprenorphine is the preferred analgesic due to its long-lasting effect and little adverse side effects. Vast differences in recommendations for dosage, application route and application intervals lead to uncertainties in the appropriate administration and counteract optimal pain management. Although strain differences in mice regarding basal pain sensitivity and the analgesic effect of other opioids e.g. morphine, fentanyl or tramadol have been described, the data for buprenorphine is incomplete.

The aim of this pilot study is to identify a dose-response curve for the analgesic effect of the opioid buprenorphine in male C57BL/6J mice using the Incremental Hot Plate test. In addition, the methods for determining the serum and brain concentration of buprenorphine and its metabolites will be established.
The dose-response curve serves to define the effective dose 50 % (ED50) and the slope of the curve. Those parameters will be used in a follow-up study which will compare the analgesic effect of buprenorphine in three different mouse strains. In addition to the analgesic effect, the serum and brain concentrations of buprenorphine and its metabolites will be analyzed to later investigate potential strain differences in phase-I and –II metabolism.

Registration details	
Status of the study	Accessible
Date of registration	2019-04-02
Notified date of accessibility	2019-04-02
DOI	10.17590/asr.0000091
Planned start of the study	2018-03-19
Planned end of the study	2018-06-30
License	

Juliane Rudeck, 2019-04-02
DOI 10.17590/asr.0000091

1. General Information

Keywords

Buprenorphine, mouse, dose-response curve

Funding sources

Bundesministerium für Bildung und Forschung (BMBF); Grant No: [031A262D]

International code of classification

Not provided

2. Study design

Introduction

The optimal dosage of analgesics should be state-of-the-art in all studies relying on animal experiments, which is not only a question of animal welfare but is also a legal provision (Directive 2010/63/EU). According to the current national and international statistics on use of laboratory animals, the mouse is the most commonly used species accounting for 70% of all animals used in research experiments. Opioid analgesics are frequently applied for perioperative care of moderate to severe pain in mice. Among the numerous opioid drugs available, buprenorphine is the preferred analgesic due to its long-lasting effect and little adverse side effects. Vast differences in recommendations for dosage (0.006 mg/kg to 10 mg/kg) [1 – 4], application route and application intervals lead to uncertainties in the appropriate administration and counteract optimal pain management. Although strain differences in the analgesic effect of opioids in mice are known, they have not been sufficiently investigated for buprenorphine, and consequently have not been taken into account in current national recommendations [1].

One reason for the vast range of recommended doses could be strain differences. Strain differences in basal pain sensitivity in mice are well known and have been described for different painful stimuli (e.g. Mogil et al. 1999 [5]). Strain differences regarding opioid sensitivity have also been shown for the opioids morphine, tramadol and fentanyl. Elmer et al. (1998), for instance, observed a 4.25-fold higher ED50 value for male C57BL/6J mice than for Balb/cJ mice [6]. Although strain differences in mice have been described for other analgesics, the data for buprenorphine is incomplete and not sufficient.

Buprenorphine is a long-acting opioid with a complex pharmacological profile. It acts as a partial μ-opioid receptor agonist, δ- and κ-opioid receptor antagonist, and opioid receptor like-1 agonist with low intrinsic activity and high receptor affinity. In humans, buprenorphine is metabolized via cytochrome P450 3A4/5 enzymes into norbuprenorphine (phase-I-reaction). Both substances are glucuronidated to buprenorphine-3-glucuronide and to norbuprenorphine-3-glucuronide and are catalyzed by UDP-glucuronosyl transferases 1A1/3 and 2B7 (phase-II reaction). The metabolite norbuprenorphine causes respiratory depression, sedation and has only a small analgesic effect. Buprenorphine-3-glucuronide exhibits a mild analgesic effect, whereas norbuprenorphine-3-glucuronide has a sedative effect and decreases tidal volume. It is assumed that orthologue enzymes to humans catalyze the same reactions in mice.

Pharmacodynamic as well as pharmacokinetic parameters might have an impact on putative strain differences in the analgesic effect of buprenorphine. The data of this pilot study, i.e. the determination of a dose-response curve of buprenorphine for C57BL/6J mice and the establishment of the method to measure serum and brain concentration of buprenorphine and its metabolites, will be used to optimally design the major follow-up study. The overall aim of the follow-up study is then to elucidate the influence of pharmacokinetic-related strain differences on the analgesic effect of buprenorphine in three different mouse inbred strains.

References:

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Type of research

Exploratory

Hypothesis of your study

The aim of this pilot study is the determination of a dose-response curve for the opioid buprenorphine and the development of the method for the analysis of buprenorphine concentrations and its known metabolites in serum and brain samples using male C57BL/6J mice. The identified parameters will be used for the optimal planning and sample size calculation of the future follow-up study.

Study design

To determine the dose-response curve of buprenorphine, the antinociceptive effect of the opioid will be assessed with the incremental hot plate test. To evaluate potential effects of buprenorphine on locomotor activity, all mice will be placed in a round open field arena for five minutes directly before the actual incremental hot plate test.

After two weeks of acclimatization on the housing conditions, each mouse will be habituated once a day to the experimental conditions in a randomized order. Therefore, mice will be fixated to mimic a subcutaneous injection and subsequently placed in a round open field arena, monitored by a video camera from above (approximately 5 minutes). Afterwards the mouse will be placed on the warm (35°C) Incremental Hot Plate for approximately five minutes, monitored by two video cameras (frontal and lateral).

At the beginning of the actual experiment, mice will be seven to eight weeks old. Directly before Incremental Hot Plate testing, locomotor activity for each mouse will be assessed in the open field test. On the first day of testing, basal pain sensitivity (temperature, T0) of each mouse will be determined. On the following day, the animal receives one dose of buprenorphine (0.05, 0.25, 0.5, 1.0, 2.0, 4.0 mg/kg; n=3 per treatment group) or 0.9 % saline to determine the antinociceptive effect of buprenorphine (T1(x)). After testing nociceptive thresholds on the Incremental Hot Plate, the mouse will be immediately sacrificed by gradual CO_2 filling and blood and brain will be taken and stored for further in vitro testing.

As the expression and activity of metabolically relevant enzymes, responsible for buprenorphine metabolism, are significantly influenced by circadian rhythm, all animal training and testing will be take place between 9.00 am to 11.00 am in winter time and 10.00 am to 12.00 at noon in summer time.

Method of blinding

The subcutaneous administration of buprenorphine, respectively, saline and the following testing will be performed and analysed by an experimenter blinded to the treatment.

Method of randomization

The order of habituation and experimental testing of all mice will be randomized with the software R using the function "rm( )".

3. Methods

3. 1. Pain assessment

Description of the method

Individual pain reaction will be investigated with the Incremental Hot/ Cold Plate Analgesia Meter for rats and mice (IITC Inc. Life Science, Los Angeles, USA, plate size 10 cm x 20 cm, 140 lux). According to the protocol by Alshahrani et al. (2012), the instrument parameters will be adjusted as follows: 35°C starting temperature, 6°C/ min heating rate and 55°C cut-off temperature. The whole test session will be documented by two video cameras, one setup in front and one at the right side of the test chamber. Cut-off criteria will be defined as

shaking or licking of one of the hind paws or jumping. Basal pain sensitivity (T0 = nocifensive temperature without analgesic) and pain sensitivity after administration of buprenorphine (T1(x)) will be determined for each animal. Both values (T0 and T1(x)) will be collected in a blinded manner by analyzing the video recordings. Following, the temperature difference ΔT = T1(x) – T0 and maximal possible effect (MPE(x) = 100% x [(T1(x) – T0) / (T2 – T0)] with T2=55°C cut-off temperature) will be calculated for each animal.

References:

S. Alshahrani, F. Fernandez-Conti, A. Araujo, M. DiFulvio, Rapid determination of the thermal nociceptive threshold in diabetic rats. *Journal of visualized experiments : JoVE*, e3785 (2012).

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Buprenorphine will be obtained as Buprenovet® (0.3 mg/ml buprenorphine, BayerHealthCare AnimalHealth, Leverkusen, Germany). Buprenorphine (0.05, 0.25, 0.5, 1.0, 2.0, 4.0 mg/kg with an injection volume of 13.5 ml/kg bodyweight) will be administered subcutaneously into the nuchal fold 30 min prior Incremental Hot Plate testing (i.e. 25 min before OF testing). For dilution or vehicle administration, sterile 0.9 % saline-solution (G-Bioscience, St. Louis, USA) will be used.

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 2. Locomotor activity

Description of the method

Locomotor activity for every mouse will be monitored for 5 min before Incremental Hot Plate testing in a round open field arena (made of green hard plastic, ø 30 cm, 40 cm height, 109 lux) filled with standard bedding material. Therefore, after application of buprenorphine the mouse will be subsequently placed in the open field arena until the Incremental Hot Plate test will start. The tracking length [cm] of the last 5 min will be analyzed with the Viewer Software, version 4 from Biobserve GmbH (Bonn, Germany) and the velocity [cm/s] will be calculated thereafter.

Narcotic/analgesic treatment

Not applicable

Drugs/substances

See method “pain assessment”.

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 3. Analysis of buprenorphine and its metabolites in serum and brain

Description of the method

Immediately after Incremental Hot Plate testing, the animals will be sacrificed by CO_2 by gradual filling and blood will be taken by heart puncture. Subsequently, the brain will be removed without bulbus olfactorius and cerebellum, weighed and instantly frozen in liquid nitrogen and stored at -80 °C. The coagulated blood will be centrifuged at room temperature for 15 min at 5,500 rcf. The serum will be removed and stored at -20 °C. Total contents of buprenorphine and its metabolite concentrations will be analyzed after protein precipitation in blood serum and brain by the MVZ Labor Dessau GmbH (Dessau, Germany) using liquid-chromatography coupled with tandem mass spectrometry (LC-MS/MS) with a detection limits of 0.1 ng/ml for all analytical targets. For quantification, isotope standards will be used with limits of quantification of 0.13 ng/ml for buprenorphine, 0.16 ng/ml for norbuprenorphine, 0.15 ng/ml for buprenorphine-3-glucuronide and 0.2 ng/ml for norbuprenorphine-3-glucuronide. To evaluate the activity of metabolizing enzymes, the metabolic ratio (MR) will be calculated for blood serum concentration data.

Narcotic/analgesic treatment

Not applicable

Drugs/substances

See method “pain assessment”.

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

4. Statistics

4. 1. See method “pain assessment”.

Assigned method(s)

Pain assessment
Locomotor activity

Analysis of buprenorphine and its metabolites in serum and brain

Main endpoints

Basal pain sensitivity (T0) and pain sensitivity after buprenorphine administration (T1(x))

Secondary endpoints

In the Incremental Hot Plate test the following additional endpoints will be assessed: straub-tail effect, frequency of defined first nocifensive behavior (e.g. licking or shacking as first nocifensive behavior). In addition, in the open field test locomotor activity in [cm] and the concentration of buprenorphine and its metabolites as [ng/ml] will be analyzed.

Sample size calculation

For the calculation of the appropriate number of animals for the dose finding study, a simulation model was set up using the software R, version 3.3.1. In this pilot study, a dose-response curve will be developed to identify the effective dose 50 % (ED50) value for male C57BL/6J mice. The identified ED50 value must be in a sufficiently precise manner to distinguish strain differences in three mouse strains (C57BL/6J, Balb/cJ, 129S1/SvImJ) in the nocifensive effect of buprenorphine by a factor of 1.5 with a power of 80 % in the major follow-up study. To develop a dose-response curve, the log10 of the administered dose will be plotted against the maximal possible effect, MPE(x) = 100% · [(T1(x) – T0) / (T2 – T0)], with T1(x): nocifensive temperature after administration of dose x, T0: nocifensive temperature without analgesic and T2: defined cut-off temperature (55 °C). The initial data for the simulation model was estimated from the literature with T0 = 47.5 °C ± 2.5 °C (mean ± sd) [1]. T1 depends on dose x and is modelled with a log logistic function: T1 = T0 + (7.5/(1-exp(b(ln(x)-ln(e))))) + ε. The increase of the log logistic function was estimated as equal for the three inbred mouse strains with b = -2 [2]. The ED50 value was presumed with e = 0.61 mg/kg for C57BL/6J [2] and e(Balb/cJ) = 2/3*e(C57BL/6J) and e(129S1/SvImJ)= 3/2*e(C57BL/6J). The variance of the normal distributed confounder ε was chosen to enable a correlation (r2) of 0.25, 0.49 and 0.81 between T1(x) and T0. The doses that will be used are 0.00, 0.05, 0.25, 0.50, 1.00, 2.00, 4.00 mg/kg. For every parameter set, 5000 dose-response relationships were simulated and adjusted with the R-function “drm” to estimate e and b. These estimated parameters were used to simulate the planned strain comparison study, using ANOVA (F-test), with a sample size of 10 to prove the initial key assumptions. This resulted in the animal number of n = 3 per dose group for the present dose-response curve finding study, with r2 =0.81, α = 0.05 and β = 0.2 (total animal number of 21 animals). References: [1] Alshahrani S, Fernandez-Conti F, Araujo A, Di Fulvio M. Rapid determination of the thermal nociceptive threshold in diabetic rats. Journal of visualized experiments: Jove 2012(63):e3785. [2] Ozdogan UK, Lahdesmaki J, Scheinin M. The analgesic efficacy of partial opioid agonists is increased in mice with targeted inactivation of the alpha2A-adrenoceptor gene. European journal of pharmacology 2006; 529(1-3):105-113.

Primary statistical analysis

The dose-response curve will be fitted with a sigmoidal 4 parameter logistic model. The analgesic effect of buprenorphine will be compared with the vehicle control group using the Kruskal-Wallis test with Dunn's multiple comparison.

Exclusion criteria

If the blinded retrospective video analysis of Incremental Hot Plate data will show that the pain assessment was stopped too early, the respective mouse will be excluded from further analysis of Incremental Hot Plate data. If the concentration of buprenorphine or

its metabolites is below the quantification limit, these samples will be excluded from the analysis.

Additional remarks

The same animals used for pain assessment will be used for the assessment of locomotion in the open field test and concentration determination of buprenorphine and its metabolites in serum and brain samples. For statistical analysis the Kruskal-Wallis test with Dunn's multiple comparison will be used.

5. Animals

5. 1. Mice (Mus musculus)

Animal strain/breed

C57BL/6J from Charles River (Sulzfeld, Germany, breeding the original Jackson strain from USA).

Genetically modified

No

Sex

Male

Further characteristics of the animals (e.g. age, body weight, size)

Male C57BL/6J mice will be obtained after weaning (three weeks old).

At the beginning of the actual experiment, mice will be seven to eight weeks old.

Housing conditions

Mice will be housed in groups of 5 to 8 animals per cage in Eurostandard type III polycarbonate cages with filter tops, autoclaved bedding and nesting material (LASbedding ^{TM}PG2, LASvendi, Soest, Germany), a mouse house consisting of board (Claus GmbH, Limburgerhof, Germany) and gnawing material (J. Rettenmaier & Soehne GmbH & Co KG, Rosenberg, Germany) as environmental enrichment. The animals will have free access to autoclaved food pellets (LASQCdiet ^{TM} Rod16, LASvendi, Soest, Germany) and acidulated water to prevent growth of pathogens. The room temperature is 21 ± 1 °C, with a relative humidity of 55 ± 10 %. The light/dark cycle in the room consists of 12/ 12 h artificial light with lights on from 5.00 am to 5.00 pm in winter time and 6.00 am to 6.00 pm in summer time. During acclimatization time, the cages will be changed once per week. Own experience has shown that male C57BL/6J mice display aggressive behavior beginning at an age of eight-ten weeks. Hence, all mice will be single housed after two weeks of acclimatization to the housing conditions. Animals will have visual and odor contact to their conspecifics. During

this time (10 days maximum) a further cage change is not necessary. All mice will be handled by hand.

Refinement

Not provided

6. Comments

2019-03-18	**Method: Locomotor acivity** Prior the Incremental Hot Plate test, the last three minutes were used for locomotion analysis to ensure a comparable time span.
2020-12-08	The data of the study are now published in Pharmacology, Biochemistry and Behavior (10.1016/j.pbb.2020.172877) and PLoSOne (10.1371/journal.pone.0230900).