Design, Characterization and In Vivo Evaluation of a Microparticulate Depot Formulation of Buprenorphine for Veterinary Use

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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Basel, 2021

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Basel, den 15. September 2020

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ACKNOWLEDGMENTS

First, I would like to thank my supervisor Prof. Dr. Jörg Huwyler for giving me the opportunity to work on this exciting project. I truly enjoyed our discussions and I thank you for your enthusiasm, encouragement and for always having an open door. I would also like to thank my second supervisor Dr. Paulin Jirkof, who provided advice, taught me all about mice and did her best to move this project forward. Furthermore, I would like to acknowledge Dr. Pascal Detampel for his supervision and guidance especially at the beginning of my PhD. I would like to express my gratitude also to Dr. Maxim Puchkov for his support, the interesting discussions about science and for his patience while trying to explain complex questions as simple as possible. Many thanks also to Denise Ruoff for the organization of many wonderful events like the ski and hiking trips and for tackling administrative challenges. I would further like to acknowledge Dr. Margarete Arras for providing the animal rooms and her support in finding collaborative partners.

Moreover, I want to thank everyone in the Pharmaceutical Technology group. Thank you Jonas Buck, Susanne Schenk, Tomaz Einfalt, Gabriela Québatte, Jan Bolten, Jens Casper, Claudio Alter and Patrick Hauswirth for the support, the laughs and the overall positive environment. A very special thank you goes to Maryam Farzan, Andreas Schittny, Roger Roth, Samuel Waldner and Darryl Borland. Thank you for all your help and for making me look forward to coming to work every single day. I would like to thank especially Darryl and Roger for reading my thesis and not being too annoyed by my “bugging”. Furthermore, I want to express my gratitude towards all the people from the practical’s for the great atmosphere, particularly Klara Kiene, Christina Häuser, Daniel Kullmann, Ariane Schmidt, Joachim Schuster and Anna Pratsinis, you all made teaching so much more fun.

Furthermore, I want to express a very special thank you to Mattea Durst, who taught me to tame mice and was an overall great help with all the animal studies.

I highly appreciated the help of Evi Bieler and Daniel Mathys from the Nano Imaging Lab for the great images from the scanning electron microscope.
I would further like to recognize all external partners of my project who supported and encouraged me. I would especially like to thank Claudia Textor for helping me to

analyze the many samples and giving me the opportunity to present my project within Novartis. I would like to express my gratitude towards Dr. Elvire Fournier from Idorsia for teaching me the techniques of microparticle production. Furthermore I would like to thank Dr. Michaela Thallmair (UZH) and the BZL staff (USZ) for their overall support of this project.

A special thank you goes to all my friends for sharing good and bad times with me. Especially my former fellow pharmacy students from Bonn, thank you for being my second family.

Above all, I would like to thank my family and especially my parents. Thank you for encouraging me to choose my path and for always believing in me. Thank you also to my aunt, uncle and grandmother for your confidence and for your help throughout my whole life.

My greatest thanks go to Jan. Thank you for your patience, your unconditional support and for being always by my side.

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SUMMARY

Buprenorphine is a semisynthetic opioid derivative commonly used to ameliorate pain in laboratory and companion animals after surgical interventions. While buprenorphine is a highly potent analgesic agent, its potency for severe side effects like respiratory depression is low. A major drawback however is buprenorphine's short terminal half-life of 3-5 hours in rodents, which necessitates repeated injections several times per day. This leads to considerable stress and pain for animals through recurring animal handling and increases workload for caretakers and researchers. Even though alternative formulations and administration routes of buprenorphine for veterinary use have been proposed, none of the suggested methods are devoid of drawbacks, side effects or other problems associated with reliable pain alleviation. The aim of this work was therefore to develop and characterize an easy to use, safe and effective depot formulation of buprenorphine to prolong the analgesic effect.

Poly (lactic-*co*-glycolic acid) (PLGA) is one of the most studied synthetic polymers for controlled release drug delivery. Its excellent biodegradable and biocompatible characteristics make it a highly valuable excipient for sustained release applications.

This work proposes therefore a novel size-controlled PLGA based microparticulate depot formulation for prolonged and controlled pain reduction. Different PLGA polymers were used to produce various microparticulate buprenorphine formulations. Characterization was done regarding size, morphology, drug load and *in vitro* release. Lead formulation was identified as a product with a burst release of roughly 30% and a controlled drug release of up to three days. Pharmacokinetic studies in naive, female, adult C57BL/6J mice revealed fast onset of action and exposure above therapeutic threshold of 1 ng/ml in plasma and brain for 12 and 72 hours, respectively sustained-release formulation was further assessed with the hotplate assay. Thereby, significant effect was shown for at least 24 hours in mice. Furthermore, analgesic effect was evaluated after sham-ovariectomy, to simulate real-life surgical set-up. Mouse Grimace Scale revealed that one injection of novel depot formulation was equivalent to several injections of commercial non-retard formulation regarding pain alleviation post-surgery. No side effects or impairments appraised by nest building behavior and clinical parameters (*e.g.* body weight, food, and water intake) were identified after

surgery. Based on the duration of action and the capability to alleviate pain reliably after surgical intervention without any side effects, this depot product is considered a valuable alternative to commercial non-retard formulations.

Further evaluation of depot formulation revealed sensitivity of buprenorphine towards X-rays during terminal sterilization process, necessitating aseptic manufacturing to ensure sterility. Further characterization in terms of potential future industrial production showed, that sustained-release formulation complies with requirements regarding bacterial endotoxin burden, residual moisture levels, shelf life after reconstitution and shelf life of final product. This work therefore indicates, that the proposed manufacturing procedures allow for industrial production and future commercialization.

A usage of buprenorphine depot formulation is therefore proposed as a safe and effective product for prolonged pain management in laboratory mice.

LIST OF ABBREVIATIONS

AT	Analgesiometric test
AU	Action unit of MGS (orbital tightening, nose bulge, cheek bulge, ear position, whisker change)
BUP	Buprenorphine
BW	Body weight
CM	Cynomolgus
DCS	Dual chamber syringe
DNA	Deoxyribonucleic acid
DoE	Duration of effect
ER	Animalgesics for Mice® from Animalgesic Laboratories Inc. (Millersville, MD)
F	Female
FI	Food intake
i.m.	Intramuscular
i.v.	Intravenous
LE	Long-Evans
M	Male
MGS	Mouse Grimace Scale
MP	Microparticles
NMP	N-methyl-2-pyrrolidone
NorBUP	Norbuprenorphine
O/W	Oil-in-water emulsion
OG	Oral gavage
OI	Orogastric infusion
PC	Plasma concentration
PGA	Poly (glycolic acid)
PLA	Poly (lactic acid)
PLGA	Poly (lactic-*co*-glycolic acid)
Ref	Reference
RGS	Rat Grimace Scale
RNA	Ribonucleic acid
s.c.	Subcutaneous
SD	Sprague-Dawley
SM	Surgical model
SR	Buprenorphine SR-Lab® from ZooPharm (Fort Collins, CO)
TD	Transdermal patch
Tg	Glass transition temperature
TW	Thermal withdrawal assay
VI-G	Voluntary ingestion in MediGel
VI-J	Voluntary ingestion in jelly
VI-N	Voluntary ingestion in Nutella®
VI-P	Voluntary ingestion in pellet feed
VI-W	Voluntary ingestion in water
W/O/W	Water-in-oil-in-water emulsion
WI	Water intake

1. INTRODUCTION

1.1 Buprenorphine

Buprenorphine (BUP, figure 1), a semisynthetic oripavine derivative, is frequently used to alleviate pain and as maintenance therapy for addiction. It was first introduced in the 1970s in the form of a solution for injection for clinical use [19]. Its high potency (25-40 times higher compared to morphine) and low physical dependence potential, made it extremely valuable [34,94]. BUP's complex pharmacology is a result of its versatile interaction with different classes of opioid receptors. Studies have shown, that BUP possesses high affinity to the major opioid receptors, classified as μ-, κ-, and δ-opioid receptors [83,194]. In contrast to morphine, which is a full μ-opioid receptor agonist, it shows mixed agonist and antagonist properties [175]. While BUP is a partial agonist for μ-opioid receptors, it shows antagonism on κ- and δ-opioid receptors [83,136,160,183,223,245]. BUP's slow receptor dissociation is responsible for its long duration of action compared to other drugs like fentanyl or morphine [17,242]. Nevertheless, its short half-life of approximately 3-5 hours in humans and rodents make it a short acting compound, limiting its antinociceptive effect to hours [20,168]. It has been suggested, that buprenorphine exhibits its analgesic effect primary through μ-receptors [77,85,106].

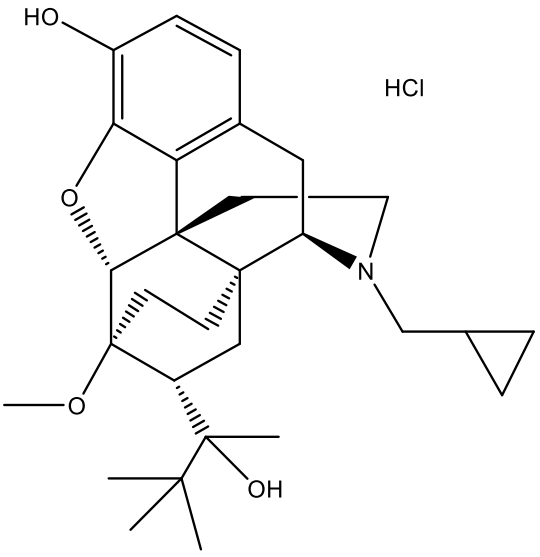

Figure 1. Chemical structure of buprenorphine HCl.

However, not only desired effects like antinociception are mediated through these receptors, but also common side effects like respiratory depression [242]. In contrast to other opioids, BUP exhibits a bell shaped dose-response curve regarding respiratory depression in humans and rodents [36,37,140]. As a result, this severe and dangerous complication, which often occurs with other opioids like fentanyl or morphine [36], is limited. BUP therefore demonstrates a high safety margin and therapeutic index, making it an extremely attractive drug for clinical use.

The ceiling effect of BUP has not only been shown for side effects, but also for analgesia in humans and rodents [34,230]. However, these studies applied very high doses far outside of the normal therapeutic range. Several other studies have therefore been conducted over the years, proving that BUP shows no bell shaped dose-response curve for analgesia in the therapeutic dose range [19,37,94].

BUP is extensively metabolized if given orally, resulting in a bioavailability of less than 20%. On the one hand, BUP is metabolized to BUP glucuronide and on the other hand dealkylated by hepatic enzymes to Norbuprenorphine (NorBUP) [32,86]. While NorBUP shows weak analgesic action compared to BUP, its effect on respiratory depression is 10 times higher than its parent drug [166,167].

1.2 Route of Administration

Due to the low bioavailability through the oral route, administration of BUP needs to bypass the enterohepatic circulation. BUP's highly lipophilic character lets it readily cross membranes, making it a good candidate for transdermal or buccal drug delivery. Moreover, its lipophilicity lets BUP also cross the blood-brain barrier easily, making it immediately available at the site of action [167].

Since its first introduction in 1979 as an injectable solution for pain management, several different formulations have been developed to prolong BUP's effect or simplify its handling [19]. Besides subcutaneous or intravenous injection, BUP can be administered via transdermal patches, especially for chronic pain treatment in humans [55]. Thereby, the drug is incorporated into a polymeric matrix, which has direct contact with the skin. BUP is released slowly and continuously over a prolonged period into systemic circulation, leading to effective pain relief for up to 7 days [11,55,120,204].

The big advantage of this route is the non-invasiveness and the easy handling of the product, which does not require additional medical personal. Furthermore, application intervals can be reduced considerable to once or twice a week, increasing patient compliance and reducing costs [11,120]. Other formulations are used to administer BUP sublingual or transmucosal and are likewise used for cancer pain or for treatment of opioid dependency [38,74,102,103]. Through continuous innovation, novel weekly to monthly depot formulations have been developed and marketed in recent years [31]. Probuphine® is a subdermal BUP implant consisting of 4 rods for continuous drug delivery over a period of 6 months. Several studies demonstrated its effectiveness for treatment of opioid dependency over 24 weeks [124,189]. One of the most recent formulations on the market is Sublocade®, which was approved in 2017 by the U.S. Food and Drug Administration. Sublocade® utilizes the sustained-release delivery technology AtriGel® and is designed to be injected once monthly [31,45,159]. It could be shown, that this formulation can be also used for addiction management [159].

1.3 Buprenorphine for Veterinary Use

BUP is not only used for pain management in humans but also to relief pain in animals, especially after surgical interventions [212]. BUP's favorable characteristics regarding potency and safety due to the occurring ceiling effect for respiratory depression, make it a widely used opioid for laboratory and companion animals [34,36,37,166]. BUP's effectiveness was demonstrated in various species (mice, rats, cats, dogs, sheep, pigs) post-surgery with varying degrees of pain [18,122,123,170,186,192,210]. It was determined, that 1 ng/ml of BUP in plasma, represents a therapeutic threshold above which an analgesic effect can be expected [241,244].

Table 1 and 2 present studies giving a statement regarding duration of action in mice, rats and dogs determined through plasma concentrations, analgesiometric tests or surgical models after parenteral application of non-retard formulations. A common dosing interval of parenteral administered BUP is twice daily. However, several studies reported that analgesic effect in animals is much shorter. In rats, a frequently used dose of 0.05 mg/kg provided plasma concentrations above or close to the presumed therapeutic threshold of 1 ng/ml for less than 2 hours or not at all [69,201].

Analgesiometric tests showed furthermore, that duration of action for this dose ranges from 1 hour to 5 hours [101,104,117,135,219]. Higher doses of 0.1 mg/kg provided in a study from Foley and colleagues concentrations above 1 ng/ml for 8 hours [60]. Likewise, Jessen et al. demonstrated that the same dose provided an effect for 8 hours using a paw withdrawal apparatus [96]. However, an even higher dose of 0.2 mg/kg given by intra muscular injection provided analgesia for only 1.5 hours in another study using male and female rats [33]. Gades et al. tested the analgesic effect in male rats with a dose of 0.5 mg/kg through a tail flick- and hot plate assay, and determined that after 6-8 hours no effect could be seen in 50% of tested animals [64]. Studies utilizing surgical models demonstrated furthermore, that a dose of 0.05 mg/kg in rats had a positive impact on body weight and water intake after catheterization, but had no effect on corticosterone levels, which were higher compared to the baseline [68,69]. Chum et al. and Seymour et al. used the plantar incision model, showing that a twice-daily injection provided sufficient analgesia, as no mechanical or thermal hypersensitivity was evident [29,201]. Another study examined rats after laparotomy and determined that animals showed lower pain scores after surgery with twice-daily 0.05 mg/kg compared to animals without analgesia. However, a dose of 0.1 mg/kg given every 12 hours showed no effect in this model [162]. The authors speculated that the higher dose led to a ceiling effect. Likewise, in a tibial defect model treated with 0.2 mg/kg rats showed fewer vertical raises compared to baseline, indicating incomplete alleviation of pain [60].

Analgesic tests and the surgical models used in these studies suggest, that the effect after single injection is less than 12 hours, indicating the need of more frequent administrations depending on the conducted surgery.

Similar findings were reported in studies with mice. Doses of 0.05-0.1 mg/kg in different strains provided therapeutic plasma concentrations of 1 ng/ml for a maximum of 6 hours [30,82,104,108]. However, a study also showed, that a dose of 0.03-0.05 mg/kg did not provide sufficient plasma concentrations at any time point [30]. Even the dose of 0.1 mg/kg twice daily did not provide sufficient analgesia in a laparotomy or cecal legation puncture model [79,109]. Furthermore, Carbone et al. could not show any efficacy at this dose in an analgesiometric test [24]. Higher doses of 1.5-2 mg/kg seem to provide longer analgesia with adequate plasma levels for 6 hours and effect for 3-5 hours [30,64,78,198]. None of the here described studies

provides any evidence, that a twice daily injection of BUP reliably alleviates pain after surgical interventions in mice. There are even some reports indicating BUP's general incapability to alleviate severe pain in rodents at all [64,65,101]. However, one has to keep in mind that pain is highly subjective and analgesic effect varies considerably depending on pain severity or pain stimulus used. Analgesic action is furthermore influenced by genetics, strain differences and sex, as has been previously shown for mice [152–154,206,207,215]. Discrepancies in findings may also be attributed to dose, route of administration and analgesiometric test used. A general statement regarding BUP's lack of effect can therefore not be made. However, looking at the literature, dosing every 12 hours does not seem to provide adequate pain relief in some surgical settings. Therefore, pain management protocols have to be evaluated carefully and dose and application intervals adjusted according to surgical intervention and resulting burden for animals.

Table 1. Duration of action of parenteral administered buprenorphine measured through plasma levels and analgesiometric tests.

Strain	Sex	Dose [mg/kg]	Duration of Effect: AT & PC	Ref
SD Rat	M	0.05	PC: < 2 h	[69]
LE Rat	M	0.05	AT: 2 h	[135]
LE & SD Rat	M	0.05	AT: 1 h	[219]
Wistar & LE Rat	MF	0.05	AT: At least 5 h	[117]
LE Rat	F	0.05	AT: Effect at 1 h	[218]
SD Rat	M	0.05	PC: Below 1 ng/ml at all time points (1, 2 ,3 d) after twice daily injection	[201]
SD Rat	M	0.05	AT: 1 h	[101]
Wistar Rat	M	0.1	AT: 8 h	[96]
SD Rat	M	0.1	AT: 4 h	[80]
SD Rat	M	0.1	PC: 8 h	[60]
SD Rat	MF	0.2*	AT: 1.5	[33]
Hsd:SD Rat	M	0.5	AT: 50% of rats had no effect after 6-8 h	[64]
C57BL/6J Mouse	M	0.03	PC: Below 1 ng/ml at all time points	[30]
NMRI Mouse	M	0.05#	PC: Both routes 6 h	[104]
C57BL/6J Mouse	M	0.05	PC: Below 1 ng/ml at all time points	[30]
C57BL/6J Mouse	M	0.1	PC: 3 h	[30]
BOMTac:NMRI Mouse	M	0.1	PC: For at least 6 h and less than 12 h	[82]
HSD:ICR Mouse	F	0.1	AT: At least 3 h	[155]
Balbc & SWR/J Mouse	M	0.1	AT: No effect	[24]
CD1 Mouse	F	0.1	PC: 2 h	[108]
Balb/c & Swiss SR/J Mouse	MF	0.5	PC: 1 ng/ml not reached at 6 h time point	[198]
Swiss Webster Mouse	M	1.5	AT: At least 4 h	[78]
C57BL/6J Mouse	M	2	PC: 6 h	[30]
Hsd:ICR Mouse	M	2	AT: 50% of animals showed no effect after 3-5 h.	[64]
Balb/c & Swiss SR/J Mouse	MF	2	PC: Above 1 ng/ml at 6 h time point	[198]
Beagle Dog	F	0.02	PC: 7 h	[164]
Mongrel Dog	MF	0.02*	PC: 2 h, AT: 12 h	[12]
Beagle Dog	MF	0.02*	PC: 2 h, AT: 6 h	[179]

All injections were administered subcutaneously (s.c.), if not stated otherwise.

**i.m. injection instead of s.c.; # s.c. and i.v. injection with same dose;*

AT = analgesiometric test (thermal or mechanical); PC = plasma concentration, duration of action is defined as the time plasma concentrations stay above therapeutic threshold of 1 ng/ml if not stated otherwise; Ref = reference; SD = Sprague-Dawley; LE = Long-Evans; M = male; F = female

Table 2. Efficacy of parenteral administered buprenorphine after surgical interventions.

Strain	Sex	Dose [mg/kg]	Effect in SM	Ref
SD Rat	M	0.05	Positive impact on BW, WI but not on serum corticosterone levels.	[68]
SD Rat	M	0.05	Higher corticosterone levels after 2, 10 h compared to oral BUP. Decreased weight gain.	[69]
SD Rat	M	0.05	Twice daily injection: Mechanical or thermal hypersensitivity after plantar incision model not different to baseline.	[29]
SD Rat	M	0.05	Twice daily injection: Mechanical or thermal hypersensitivity after plantar incision model not different to baseline.	[201]
SD Rat	F	0.05 0.1	Laparotomy: No effect at 0.1 mg/kg twice daily. 0.05 mg/kg showed lower pain scores compared to control without analgesic treatment.	[162]
SD Rat	M	0.2	Tibial defect model: Fewer vertical raises compared to baseline on day 1-3.	[60]
Crl:CD1 Mouse	F	0.1	Twice daily after laparotomy did not provide adequate analgesia.	[109]
C57BL/6J Mouse	M	0.1	Cecal legation puncture model: effect less than 6 h.	[79]
Beagle Dog	F	0.02	Twice daily is efficacious after ovariohysterectomy to manage pain.	[164]
Dog	F	0.02	Twice daily is efficacious after ovariohysterectomy to manage pain.	[156]

All injections were administered subcutaneously.

SM = surgical model; Ref = reference; SD = Sprague-Dawley; M = male; F = female; BW = body weight; WI = water intake

1.3.1 Available Formulations and Alternatives

A further complicating factor in pain management is the lack of alternative BUP formulations for veterinary use. As of now, only immediate acting injection solutions are available in Europe. As a result, repeated administrations per day are necessary to relief moderate to severe pain after surgeries reliably [64,243]. In this way however, animals experience additional stress through recurring injections and animal handling [141]. Other possibilities to treat animals post-surgery include alternative opioids like fentanyl and morphine, which proved to be successful in pain alleviation in different species [27,64,66,113,137]. However, these opioids show an inferior safety profile, endangering animals through side effects like respiratory depression [36,105,187]. Moreover, duration of action is much shorter, excluding them as possible choice for standard pain treatment after surgical interventions [64,66,88]. Although non-steroidal anti-inflammatory drugs (NSAIDs) can be also used, their application as single agent is not recommended for post-surgical pain relief, as alleviation of severe pain is not possible [190,201,228].

1.3.1.1 Oral Buprenorphine

A summary of studies using oral BUP in various rat and mice strains can be found in table 3 and a more detailed version in the supplement (table S1). Although BUP shows low bioavailability if delivered orally, its effectiveness has been shown in rodents if delivered through this route. However, this fact makes it necessary to add high amounts of drug to eatables to reach analgesic effect. Several studies demonstrated that an approximately 10 times higher dose (0.3-0.5 mg/kg) of oral BUP in rats is comparable to the standard subcutaneous dose of 0.05 mg/kg [58,68,123]. But it was also shown, that this dose is not sufficient in rodents and an approximately 100 times higher dose (5 mg/kg) of oral BUP was required to induce robust analgesia [33,135,218,219]. High drug concentrations however, may result in a bitter taste, leading to refusal of fodder or water by animals [219]. Jessen et al. demonstrated that consumption of drug-spiked water was reduced after the first day, which could be due to the bitter taste or gastric distress [96]. Thompson et al. had similar results, showing

that oral BUP but also BUP administered through subcutaneous injection induced a measurable aversion to grape juice [219]. Martin et al. showed furthermore, that high drug concentrations in flavored gelatin, a readily consumed item, led to unpalatability at the required dose of 5 mg/kg [135]. BUP has therefore measurable negative effects on ingestion and these findings have to be taken into account, if rodents are treated orally. The summarized studies in table 3 and table S1 show the prospect of voluntarily ingestion of drug through food or water [82,123,135,197,215]. Thereby, animals can be either trained to ingest a specific amount of drug laced nut paste or flavored gelatin at a specific time or the food and water can be left in the home cage for continuous consumption. Both approaches provide overall adequate plasma levels and measurable effect [33,82,96,104,135]. However, common side effects of surgical interventions and opioids include reduced food and water intake [58,87,121]. In addition, ingestion is often coupled to circadian rhythm, resulting in considerable less water consumption during the day. Adequate plasma levels of BUP can therefore not be guaranteed for individual animals at all time points [54,197]. Neophobic behavior of rodents can further impede effective treatment. Animals have to be trained for several days before they reliably eat their assigned share or drink the spiked water [209]. However, an exposure to the drug or vehicle for several days preceding surgery is not always possible. Potential side effects of BUP would also persist for a longer time in the case of habituation to drug-laced water. Furthermore, if food or water is left in the home cage and consumption is not monitored, there is no control over the ingested amount and period [82]. Monitoring becomes even more difficult if animals are group housed. Unmonitored consumption can even lead to an overdose. Duration of action and dosing interval are other important parameters that need to be evaluated. Goldkuhl et al. could show, that rats achieved therapeutic plasma concentrations above or close to 1 ng/ml for 14 hours after ingestion of 0.4 mg/kg BUP in nut paste [69]. Kalliokoski et al. had similar findings with therapeutic plasma concentrations for at least 12 h in male mice after ingestion of the same dose [104]. Antinociceptive tests in rats at a dose of 0.5 mg/kg however, provided no effect at any time point. There, only doses of 5 mg/kg and higher provided measurable effect that persisted for 4-8 hours [135,218,219]. Interestingly, Leach et al. demonstrated that effect in thermal nociceptive test for up to 5 hours in rats could only be measured for a dose of 0.5 mg/kg through oral gavage. Voluntary ingestion of same dose in nut paste, resulted only in a

measurable effect at the 1 hour time point [117]. This demonstrates, how variable and hard to control voluntary ingestion of drug-laced food is. Another concern is the development of tolerance leading to decreased analgesic effect. Jessen et al. speculated, that male rats responded less to BUP after dosing through drug laced water over several days despite continuous intake [96]. The question arises, which administration schedule provides the most reliable analgesic effect over time. Sauer et al. could show, that the most effective pain treatment in mice after surgery consisted of an initial three subcutaneous injections (0.1 mg/kg) every 4 hours and subsequent BUP laced water (1 mg/kg/d). All individual plasma concentrations reached 1 ng/ml for 18 hours [197]. Goldkuhl and colleagues investigated the corticosterone levels of male rats after catheterization and compared a 0.05 mg/kg subcutaneous injection every 8 hours with a dose of 0.4 mg/kg of voluntarily ingested nut paste. It could be shown, that control and orally treated animals showed significantly lower corticosterone levels compared to the animals receiving injections, demonstrating the validity of the approach to minimize handling associated stress through oral administration. However, both treatments still decreased weight gain in rats [68,69]. Liles et al. showed also that a dose of 0.5 mg/kg voluntarily ingested BUP still led to reduced bodyweight and water and food intake, hinting at ineffective treatment after laparotomy [123]. It is not clear, whether the used dose of BUP in this studies was not high enough, or if body weight reduction is not a useful parameter to assess pain relief. Most studies conducted with oral administered BUP are either pharmacokinetic studies or studies measuring mechanical or thermal induced antinociceptive effect [82,96,104,135,215,218]. While both approaches are legitimate and can give valuable information on duration of action and potency of an analgesic, it is still necessary to test the formulation in a surgical setting. Therefore, protocols for oral pain relief have to be adjusted regarding dose and application interval depending on expected burden for animals.

Table 3: Efficacy of oral administered BUP formulations in rats and mice.

Strain	Sex	Dose [mg/kg]	Route	Method	Result & Reference
Wistar Rat	F	0.1-0.4	VI-J	SM-Lap	All doses prevented decrease in BW and WI. FI was still decreased [58].
SD Rat	M	0.4	VI-N	SM	Oral treatment showed lower corticosterone levels after surgery compared to s.c. injection [68].
SD & Wistar Rat	M	0.4	VI-N	SM, PC	Oral treatment showed lower corticosterone levels 10 h after surgery compared to s.c. injection. Treatment decreased BW loss compared to control. DoE VI-N: 14 h [69].
SD Rat	MF	0.3-0.6	VI-N	TW	Male rats showed effect at a dose of 0.5 & 0.6 mg/kg 1 h after treatment. Female rats showed no effect at all doses [215].
LE Rat	F	0.5	VI-W	TW	No effect at a dose of 0.5 mg/kg [218].
Wistar Rat	M	0.5	VI-J	SM-Lap	BUP decreased effects on BW, WI after surgery, but all parameters were still reduced [123].
Wistar & Lewis Rat	MF	0.5	VI or OG	TW	DoE OG: At least 5 h. DoE VI: 1 h [117].
SD Rat	M	0.5-2	VI-N	TW	0.5 mg/kg: No effect. 2 mg/ kg: effect only at 2 h time point DoE 1 mg/kg: 1-2 h [80].
SD Rat	MF	0.6-2.9	VI-W	TW	Only 2.9 mg/kg lead to measurable effect after 24 h [33].
LE & SD Rat	M	0.5-5	OI	TW	No effect at 0.5 mg/kg. DoE 5 mg/kg: 4 h [219].
LE Rat	M	0.5-10	OI	TW	0.5 mg/kg: No effect. DoE 5-10 mg/kg: 4 h-8 h [135].
LE Rat	M	0.5	VI-J	TW	No effect [135].

Table 3: Continued.

Strain	Sex	Dose [mg/kg]	Route	Method	Result & Reference
Wistar Rat	M	2.4-4.8	VI-W	TW	DoE for BUP in water for 3 d: 63 h [96].
Wistar Rat	M	0.1 + 3.9-5.6	s.c. + VI-W	TW	DoE for BUP s.c. + in water for 2 d: 39 h [96].
NMRI Mouse	M	0.4	OG or VI-N	PC	DoE OG or VI-N: At least 6-12 h [104].
NMRI Mouse	M	1-3	VI-G	PC	DoE after 15 h of ingestion for 1 mg/kg/d: 6 h. For 3 mg/kg/d: 8-12 h [82].
Hsd:ICR Mouse	F	0.75-4.2	VI-P	TW	All treatments effective compared to s.c. 3 h post injection [155].
Hsd:ICR Mouse	F	0.1 + 0.79	s.c.+ VI-P	TW	All treatments effective compared to s.c. 3 h post injection [155].
Hsd:ICR Mouse	F	0.1 + 0.3	s.c. + VI-P	SM	Clinical improvement compared to vehicle [155].
C57BL/6J Mouse	F	1	WI	PC	Individual mice showed PC below therapeutic threshold for all time points [197].
C57BL/6J Mouse	F	0.1 + 1	2x/3x s.c. + VI-W	PC	Individual mice showed PC below therapeutic threshold for all time points [197].
C57BL/6J Mouse	F	0.1+ 1	3x s.c. + VI-W	SM	Effective pain treatment [197].

SD = Sprague-Dawley; LE = Long-Evans; M = male; F = female;

s.c. = subcutaneous injection; i.m. = intramuscular injection;

VI-N = voluntary ingestion in Nutella; VI-P = in pellet feed; VI-W = in water; VI-J = in jelly; VI-G = in MediGel; OI = orogastric infusion; OG = oral gavage;

SM = surgical model; TW = thermal withdrawal assay; WI = water intake; FI = food intake; BW = body weight; PC = plasma concentration, duration of action is defined as the time plasma concentrations stay above therapeutic threshold of 1 ng/ml if not stated otherwise; DoE = duration of effect

1.3.1.2 Transdermal Buprenorphine

Additionally, the use of transdermal patches for animals was investigated and adequate plasma levels of BUP were found in pigs and dogs [4,179,216]. Table 4 and table S3 show a summary of studies in dogs, cats and pigs treated with transdermal patches. The present studies are all conducted in bigger animals, showing a great disadvantage of the available patch systems. The size of the patch is approximately 50 cm^2, which requires a big contact area [67]. The patches are therefore not applicable for animals like mice and rats due to their small body size but also because of the danger of patch ingestion. Furthermore, for acceptable adhesion, fur of animals has to be shaved and patches have to be secured with bandages to prevent loss [4,158,216]. And even if skin contact is ensured, some animals showed no detectable BUP plasma concentrations [216,179]. It was speculated, that hair regrowth was responsible for an absence of sufficient patch-to-skin contact. Beyond that, localized skin reactions were observed in studies with pigs [216]. Another limiting factor to consider is the delayed onset of action. Pieper and colleagues demonstrated in Beagle dogs, that therapeutic plasma levels of around 1 ng/ml were only reached after 48 hours and effect could only be measured after 36 hours in an thermal antinociceptive test with a 52.5 µg/h patch [179]. Andaluz et al, showed likewise in dogs, that therapeutic plasma levels were only reached after 36 hours, even though the patch had a higher dose with 70 µg/h [4]. These findings suggest that effective pain control can only be achieved if patches are administered at least 48 hours before any painful procedure. Moll et al. followed this approach in their study with female dogs undergoing ovariohysterectomy. All patches with a dose of 70 µg/h were attached 48 hours before surgery and demonstrated significantly lower pain scores after surgery compared to the control group without analgesics [156]. A study in cats receiving a dose of 35 µg/h revealed that this lower dose was not sufficient to produce a measurable effect in a thermal withdrawal assay, although presumed therapeutic levels of 1 ng/ml were reached after 22 hours [158]. It seems that the dose for effective pain management through transdermal patches in cats and dogs need to be at least 52.5 µg/h and attachment needs to be done 48 hours before surgical intervention.

Table 4: Efficacy studies of transdermal buprenorphine.

Strain	Sex	Dose	Test Method	Result & Reference
Beagle Dog	MF	52.5 µg/h	TW, PC	TD: 1 ng/ml was reached after 48 h and remained there until patch removal. Measurable effect: 36 h-72 h [179].
Beagle Dog	M	70 µg/h	PC	PC increased during first 36 h and remained at 0.7-1 ng/ml for 72 h [4].
Dog	F	70 µg/h	SM	TD patch effectively alleviated pain [156].
Göttingen Minipgs	F	30 µg/h	PC	Hypothesized therapeutic threshold: 0.1 ng/ml. DoE TD: Threshold is reached after 12-24 h. Remains there until 72 h [216].
Shorthair Cat	MF	35 µg/h	PC, TW	TW: no effect at any time point. PC: 1 ng/ml was reached after 22 h [158].

M = male; F = female; TD = transdermal patch; PC = plasma concentration; TW = thermal withdrawal assay; SM = surgical model; DoE = duration of effect

1.3.1.3 Sustained-release Buprenorphine

Recently, BUP depot formulations for veterinary use have been introduced to the U.S. market. Animalgesics for Mice & Rats® (ER, former from Animalgesic Laboratories Inc., Millersville, MD now Ethiqua XR®, Fidelis Pharmaceuticals, North Brunswick, NJ) consists of cholesterol, glycerol tristearate and medium-chain triglyceride oil and is administered subcutaneously as a suspension [70,71,139]. Likewise, Buprenorphine SR-Lab® (ZooPharm, Fort Collins, CO) is administered subcutaneous as polymeric in-situ forming implant [60]. Especially Buprenorphine SR-Lab® (SR) has been extensively studied in a variety of animals including rats, mice, guinea pigs, cats, dogs, sheep, macaques and even more unusual animals like prairie dogs, elephant seals and kestrels [24–26,60,73,157,163,164,169,205,222,229]. A summary of all studies investigating efficacy of the mentioned sustained-release formulations can be found in table 5 and a more detailed version in the supplement in table S2. SR was tested in

rats in different concentrations ranging from 0.3-4.5 mg/kg. Doses of 0.3-1.2 mg/kg seemed to achieve promising results for several days [29,60,201]. Seymour et al. demonstrated that after a single subcutaneous injection of 1.2 mg/kg SR in a plantar incision model, thermal and mechanical hypersensitivity were not significantly different to baseline animals for 96 hours [201]. Foley and colleagues analyzed plasma of male rats after injection of 0.9 and 1.2 mg/kg and detected drug levels above or very close to 1 ng/ml for at least 24 hours. Effect was further assessed through analgesiometric tests and a tibial defect model, suggesting pain alleviation for 72 hours [60]. Higher concentrations of drug in rats (4.5 mg/kg) did not produce longer analgesia, but reduced body weight of animals clinically and led to severe sedation [29]. Thereby, a dose of 1.2 mg/kg in rats seems to be the best choice regarding sufficient analgesia and absence of side effects. In contrast to rats, a dose of 0.3 mg/kg SR did not provide therapeutic plasma levels in male C57BL/6J mice. Only a dose of 1.2 mg/kg SR achieved therapeutic levels for 12 hours [30]. However, studies in CD-1 mice with half of the dose (0.6 mg/kg) produced plasma levels of around 1 ng/ml for 24-48 hours and alleviated pain after laparotomy for at least 12 hours [108,109]. A higher dose of 1.0 mg/kg led likewise to a measurable effect in an antinociceptive test for 12 hours [24]. Herndon and colleagues used the same dose in male C57BL/6 mice after a cecal ligation and puncture model, and showed a similar duration of action of 24 hours [79]. Further increase in dose to 1.5 mg/kg produced a longer effect up to 48 hours in Swiss Webster mice [78]. Although pain management of 72 hours is not reached with the investigated doses, pain alleviation after single injection with a dose of 0.6-1.2 mg/kg for 24 hours seems possible in mice. Companion animals like cats and dogs have also been tested with the novel sustained-release formulation. In cats, a dose of 0.12 mg/kg SR pre-emptive to ovariohysterectomy did not induce hypersensitivity after 12-72 hours post-surgery compared to baseline animals, indicating the effectiveness during that period of time [26]. Still, the first hours after surgery are the most painful and analgesia should be effective especially during that time. Female dogs injected with a dose of 0.2 mg/kg showed therapeutic plasma concentrations up to 72 hours post injection and after ovariohysterectomy pain scores were reduced at all time points [164]. Sheep showed a different pharmacokinetic, as doses of 0.05-0.27 mg/kg SR reached therapeutic plasma levels only after 48 hours and remained there for 72-192 hours. Effect measured in an thermal withdrawal test lasted from 12 hours to

72 hours [229]. Thereby, sheep have to be injected 24-48 hours prior to surgical interventions with SR.

Table 5: Efficacy studies of parenteral sustained-release buprenorphine formulations.

Strain	Sex	Dose [mg/kg] / Formulation	Duration of Effect & Reference
Sprague-Dawley Rat	M	0.3 / SR	AT: 48 h [29].
Sprague-Dawley Rat	M	0.9 / SR	PC: 24-48 h [60].
Sprague-Dawley Rat	M	1.2 / SR	PC: 24-48 h. AT: 72 h compared to within group baseline. But significant effect only at 48 h time point compared to saline animals [60].
Sprague-Dawley Rat	M	1.2 / SR	AT: At least 72 h [29].
Sprague-Dawley Rat	M	1.2 / SR	PC: Above threshold only at 48 h. AT: 96 h [201].
Sprague-Dawley Rat	M	1.2 / SR	AT: Effect from 24-72 h compared to baseline but no effect if compared to saline animals [101].
Sprague-Dawley Rat	F	1.2 / SR	PC: Only at 24 h time point above threshold [162].
Sprague-Dawley Rat	M	4.5 / SR	Severe sedation, BW clinically reduced. AT: At least 72 h [29].
C57BL/6J Mouse	M	0.3 / SR	PC: Therapeutic levels were not reached [30].
CD-1 Mouse	F	0.6 / SR	PC: 24 h [108].
BALB/c & SWR/J Mouse	M	1.0 / SR	AT: Effect at 2, 6 and 12 h [24].
C57BL/6J Mouse	M	1.2 / SR	PC: 9-12 h [30].
Swiss Webster Mouse	M	1.5 / SR	AT: Effect at 4, 24, 48 h compared to control [78].
Guinea pig	F	0.3 / SR	PC: Above 0.5 ng/ml for 26 h. AT: Effect to within group baseline from 6-48 h [205].

Table 5: Continued.

Strain	Sex	Dose [mg/kg]/ Formulation	Duration of Effect & Reference
Dorset & Suffolk Sheep	F	0.05 / SR	PC (0.1 ng/ml): From 48-72 h [249]
Dorset & Suffolk Sheep	F	0.1 / SR	PC (0.1 ng/ml): From 48-192 h [249]
Suffolk Sheep	MF	0.27 / SR	Comparison of i.m. with s.c. PC: Similar for both. Therapeutic levels (0.5-0.7 ng/ml) reached only after 48 h. AT: 12-72 h [229].
Beagle Dog	F	0.2 / SR	PC: 72 h [164].
Shorthair Cat	F	0.12 / SR	AT: Higher hypersensitivity scores during recovery from anesthesia. But no difference to within group baseline 12-72 h post-surgery [26].
Cynomolgus & Rhesus Macaques	M	0.2 / SR	PC: More than 60 h [163].
Prairie Dog	MF	0.9-1.2 / SR	PC: Reached between 0-4 h and maintained for 96 h [25].
Elephant Seal	MF	0.12 / SR	PC: Reached after 12 h and maintained for 24 h [157].
American Kestrel	MF	1.8 / SR	Comparison of i.m. with s.c. PC: 48 h for both routes [73].
Sprague-Dawley Rat	M	0.65 / ER	AT: Effect at 4-48 h compared to within group baseline and saline animals [101].
Fischer Rat	MF	0.65 / ER	PC: 48 h. AT: Higher latencies for 5 d [70].
Fischer Rat	MF	1.35 / ER	PC: 96 h. AT: Higher latencies for 5 d [70].
BALB/c Mouse	MF	3.2 / ER	PC: 72 h [222].
Guinea Pig	F	0.48 / ER	PC: From 24 to 48 h. AT: Hypersensitivity at 32 h and 96 h [169].
Mongrel Dog	MF	0.2 / ER	PC: 72 h. AT: From 1 h to 72 h [12].

SR = Buprenorphine SR-Lab® from ZooPharm (Fort Collins, CO); ER = Animalgesics for Mice® from Animalgesic Laboratories Inc. (Millersville, MD); M = male; F = female; PC = plasma concentration, duration of action is defined as the time plasma concentrations stay above therapeutic threshold of 1 ng/ml if not stated otherwise; AT = analgesiometric test, can stand for thermal and/or mechanical withdrawal assays.

Although SR demonstrates its usefulness in prolonged pain alleviation in a variety of animals, a big concern turns out to be the regularly seen reactions at the site of injection. In most studies and with most tested animals, reactions after subcutaneous administration are reported, ranging from mild erythema to full necrosis [24–26,30,60,157,162,163,205,249]. Page et al. reported that all immunodeficient rats treated with SR showed nodules at injection site three months after administration. They speculated, that these rodents were not able to break down the polymer subcutaneously and that drug could therefore not be released [171]. The immune system seems to plays a role in polymer degradation, hence usage in immunodeficient animals is not recommended. Furthermore, for small animals where small application volumes are required, difficulties in handling have been reported. The solution seems to be highly viscous, leading to problems regarding aspiration and injection [24,201]. Therefore, administration of SR needs to be considered carefully and animals have to be monitored for side effects at the injection site.

Even though ER formulation was not studied as extensively, its efficacy could be shown in several animals like mice, rats, guinea pigs and dogs [12,72,101,169,198,222]. In rats, doses of 0.65 mg/kg provided therapeutic plasma levels for 48 hours and measurable effect for 48-120 hours [70,101]. In mice, a dose of 3.2 mg/kg showed plasma levels above 1 ng/ml for 72 hours [222]. The high dose for mice, which is also recommended by the supplier, is surprising. The competitor's dose of BUP SR for mice corresponds to only 1.0 mg/kg. It is not clear, whether the prolonged effect and exposure of ER in mice is achieved through the formulation or the 32 times higher dose.

Although both formulations present valuable alternatives in pain management, the fact remains, that these products cannot be purchased in or imported to the European Union. Only U.S. veterinarians have the access to both formulations so far. Given the unfavorable side effect profile of SR and the required high doses of ER, both formulations present a good but not optimal solution to the current problem.

1.4 Pain Assessment in Animals

Assessment of pain and thereby analgesic effect in animals is difficult. Even in humans, where effect can be assessed through questionnaires, variability in response to analgesics is high. Analgesic effect in general can be influenced on the one hand by pharmacokinetics, meaning distinct differences in absorption, distribution, metabolization and excretion from one individual to another. On the other hand, analgesic effect is also depending on the type and severity of pain, test method used or the placebo effect [3,112]. As already mentioned above, duration and magnitude of analgesic effect in mice was shown to be also influenced by sex and genetics [154]. Moreover, rodents are prey animals and tend to not obviously show signs of impairment. Pain assessment in these animals can therefore be more challenging [211]. Determination of analgesic effect in animals can be roughly summarized in three groups: (1) Pharmacokinetic studies with subsequent correlation of plasma levels to effect. (2) Analgesiometric tests utilizing thermal, mechanical, chemical or electrical stimuli to determine analgesic potency. (3) Surgically or otherwise induced tissue or nerve injury models, with subsequent analysis of impact on behavior and physiological and clinical parameters.

1.4.1 Pharmacokinetic Studies

Pharmacokinetic studies can be helpful to evaluate the route of application and determine how fast a drug reaches adequate plasma levels or concentrations in other target tissues. However, correlation of pharmacokinetics in plasma and pharmacodynamics data is not always straightforward. Ohtani et al. injected male Wistar rats with 8 µg/kg BUP intravenously and analyzed blood and brain concentrations, as wells as analgesic effect through a tail flick test. Plasma levels were close to the threshold of 1 ng/ml for 1 hour and specific binding concentrations in the brain for approximately 3 hours. An increase in latency could be detected up to 4 hours post-injection. The investigation of the relationship between concentration and analgesic effect revealed that the best correlation could be obtained between effect

and specific binding concentrations in the brain. Thereby, analgesic effect is closely related to the concentration of drug at the target site but not of drug in plasma [167]. Another study in humans investigated plasma concentrations after application of a transdermal BUP patch with a dose of 35 µg/h and tried to correlate them to analgesic effect measured through three different analgesiometric tests and pupillometry. BUP showed significant effect compared to placebo at 24 and 72 hours in two of the analgesiometric tests and in the pupillometry. Plasma concentrations were around 0.25 ng/ml for the same time points. No significant correlation could be established between the effect and the plasma concentrations. The authors speculated that the lack of correlation was due to the high individual variability regarding responsiveness [112]. It would be interesting to know, if better correlation could be established between effect and brain concentrations in this study similar to the rat study by Ohtani et al. These studies demonstrate convincingly, that correlation of plasma levels and analgesic effect is not always possible and also that the often described threshold of 1 ng/ml might not be conclusive. Therefore, statements about magnitude and duration of effect after BUP administration must not be based solely on pharmacokinetic studies.

1.4.2 Analgesiometric Assays

Another recognized way to establish analgesic efficacy is by utilizing analgesiometric tests as described above. Here, analgesic potency is assessed by determining drug induced changes in reaction latency to thermal, mechanical or chemical noxious stimuli. However, while these assays provide a convenient ways to measure the effect without the induction of lasting harm like tissue trauma, dosage recommendations based on these tests alone are controversial [192]. Objections refer to the differences between nociception threshold, which is measured in analgesiometric assays, and clinical pain resulting from surgeries, injuries or diseases. The underlying neurological mechanisms for both conditions are very different. It could be shown, that withdrawal responses to noxious stimuli as applied in nociceptive tests, can be unconscious reflexes and protective responses without the involvement of the brain, where pain is

normally perceived [116,188]. Moreover, obtained results from analgesiometric tests are highly variable as summarized by Roughan and Flecknell [192]. The authors explain these observed differences by subdividing pain in two classes: phasic and tonic nociception. Phasic pain is thereby short-lived as in the hot plate assay, where only nociceptive threshold can be measured. While tonic pain lasts for long periods as seen in the formalin test and has distinct pain signal transduction and processing [192]. Furthermore, a treatment with a noxious stimulus tested by different assays can have opposing outcomes as shown by Autier and colleagues. In this study, male Sprague-Dawley rats were injected with Taxol®, which produces painful peripheral neuropathy in humans. Subsequent analgesiometric tests revealed, that the drug produced on the one hand mechanical hyperalgesia and on the other thermal hypoalgesia [10]. The multiple mechanisms of pain and multitude of described nociceptive assays make it difficult to rely solely upon analgesiometric tests when giving dosage recommendations [62,192].

1.4.3 Surgically or Otherwise Induced Tissue or Nerve Injury Models

Surgical models reflect certainly most closely the experienced pain by laboratory animals. However, ethically it is difficult to subject healthy animals to sham-surgeries to assess the produced pain afterwards. Nevertheless, testing analgesic compounds in real-life surgical settings is important to assess additional clinical parameters and side effects. For example, Chum et al. dosed male rats in their study with 4.5 mg/kg SR and measured hypersensitivity in an incision pain model. This dose provided sufficient analgesia for at least 72 hours in the used assays. However, severe sedation was additionally observed and body weight was clinically reduced [29]. Generally, pain assessment after surgical intervention is done by measuring the change in effects produced by said surgery with and without analgesic treatment or compared to other appropriate control groups [68,69,162,72,79,109,164]. Here again, experimenters have to consider ethically if control groups without analgesic intervention are necessary. Pain can be thereby assessed by clinical parameters and pain scoring systems. Post-surgical impairment is frequently appraised by clinical or behavioral

changes like reduction in body weight, water and food intake, locomotor activity, burrowing, and change in nesting behavior [29,58,68,69,97,98,100,101,123,162,197]. However, administration of BUP or subjecting animals to anesthesia or a combination of both, can have distinct impact on several of these parameters [81,121,144]. Another approach to assess pain in rodents is through change in facial expressions, with the help of the so called Mouse/Rat Grimace Scale (MGS/RGS) [28,111,115,138,144–146]. Hereby, mice and rats are placed in small transparent Plexiglas® containers and filmed for several minutes. Several pictures are generated out of the filmed material, and mice expressions are scored depending on the intensity of their action units (AU = orbital tightening, nose bulge, cheek bulge, ear position, whisker change). The scoring is done for every AU, whereby a 0 represents the absence of this AU, a 1 stands for a moderately visible AU, and a 2 stands for a severe pronounced AU. The average of all AUs represents the pain score for every individual mouse [115,142]. The big advantage of this technique is the measuring of spontaneous behavior, without the presence of an experimenter. Animals can be furthermore used as their own baseline, there is no “behavioral tolerance” due to habituation to the test procedure, experimenters can be blinded to treatment, the procedure does not harm the animals or induce further pain, several studies showed its accuracy and reliability and observers need minimal training to perform it [111,115,138,146]. However, it was shown that anesthesia alone can influence the MGS or RGS [144,145,147]. This demonstrates again, how important appropriate control groups are, depending on the research question. Therefore, to determine reliably analgesic effect after surgery in laboratory animals, different types of measurements have to be done and a multitude of different parameters have to be evaluated and compared with relevant control groups.

In our studies, we used versatile methods to assess analgesic effect of different BUP formulations in female C57BL/6J mice. On the one hand, we determined pharmacokinetics in plasma and brain of retard and non-retard formulations. On the other hand, we used one of the most frequent utilized analgesiometric tests, the hot plate assay, and compared both formulations with a saline control group. Furthermore, analgesic effect was investigated after sham-ovariectomy and pain alleviation of depot formulation was compared to the gold standard analgesic protocol utilizing non-retard

formulation. We therefore combined results from all groups of measurements to be able to give a reliable statement regarding efficacy.

1.5 PLGA Based Controlled Release Formulations

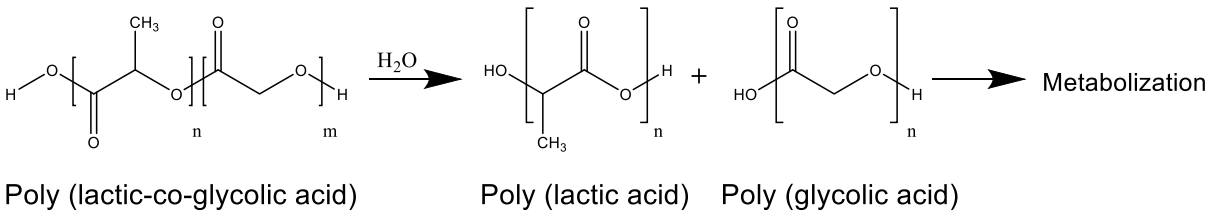

Figure 2. Hydrolysis of PLGA to poly (lactic acid) and poly (glycolic acid) and subsequent metabolization.

Parenteral controlled release formulations offer many advantages over conventional treatment regimes. Immediate acting formulations like the previously discussed non-retard formulation of BUP result in a sharp increase of drug concentration in plasma, followed by a fast decline within a few hours to sub-therapeutic levels [20,167,236]. Reinjections are therefore indispensable to achieve sufficient high plasma concentrations and thereby therapeutic effect. A sustained-release formulation would enable more stable drug levels over a prolonged period of time, reducing not only side effects through avoidance of drug plasma peaks but also injection intervals. Animals as well as caretakers and experimenters would profit alike from such a depot formulation. Such controlled drug delivery systems however, have to be made from a suitable vehicle. In recent years, advanced delivery vehicles were developed to prolong not only release of small molecules, but also of proteins, peptides, RNA and DNA [6,110,172,220,235,246]. Thereby, synthetic polymers such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymer PLGA received tremendous attention due to their biocompatible characteristics. However, not only the nontoxicity of these polymers is outstanding. Especially their excellent biodegradability makes them a sought-after excipient [5,47,132]. Once in contact with an aqueous phase, the polymeric backbone of PLGA is hydrolyzed into oligomers and monomers, with subsequent metabolization and elimination from the body (figure 2) [5,90,203]. The big advantage is hereby, that degradation of the polymer and the consequently drug release can be precisely regulated. Thereby, sustained-release can be achieved from

days to months by varying polymer properties like molecular weight, ratio of lactic to glycolic acid or polymer end-group [9,129,174,231,233,240]. Especially PLGA has been extensively studied in the past decades and received approval for parenteral application in humans by the U.S. Food and Drug Administration and by the European Medicine Agency. Table 6 summarizes the so far approved PLGA based formulations for clinical use in humans.

Table 6. Approved PLGA formulations for clinical use in humans.

Tradename & Active Agent	Delivery System	Polymer	Interval	Indication	Ref
Arestin® Minocycline	MP in cartridge	PLGA	-	Periodontitis	[7]
Bydureon® Exenatide	Vial with MP	50:50 PLGA	1 week	Type 2 Diabetes	[22]
Decapeptyl® Triptorelin	Vial with MP	PLGA	3-6 months	Prostate & Breast Cancer, Endometriosis	[30, 31]
Eligard® Leuprolide Acetate	AtriGel® in-situ forming implant	PLGA	1-6 months	Prostate Cancer	[193]
Enantone® Leuprolide Acetate	DCS with MP	PLGA	1 month	Prostate Cancer	[48]
Lupron Depot® Leuprolide Acetate	DCS with MP and diluent	PLGA PLA	1 month 3-6 months	Prostate Cancer	[130, 177]
Nutropin Depot® Somatropin	Vial with MP	PLGA	1-2 months	Growth Failure	[165]
Risperdal Consta® Risperidone	Vial with MP	75:25 PLGA	2 Weeks	Schizophrenia	[185, 184]

Table 6. Continued.

Tradename & Active Agent	Delivery System	Polymer	Interval	Indication	Ref
Salvacyl® Triptorelin Pamoate	Vial with MP	PLGA	3 months	Sexual deviation in adult men	[195]
Sandostatin® LAR Octreotide Acetate	Vial with MP	PLGA	1 month	Acromegaly	[196]
Signifor® LAR Pasireotide	Vial with MP	PLGA 50:50	1 month	Acromegaly	[202]
Somatuline LA® Lanreotide	Vial with MP	PLGA	2 weeks	Acromegaly	[114]
Sublocade® Buprenorphine	AtriGel® in-situ forming implant	PLGA	2 weeks/ 1 month	Opioid Use Disorder	[213]
Suprefact Depot® Buserelin Acetate	Pre-filled syringe with implant rods	75:25 PLGA	2-3 months	Prostate Cancer	[214]
Trelstar® Triptorelin Pamoate	Vial with MP	PLGA	1-6 months	Prostate Cancer	[224]
Triptodur® Triptorelin	Vial with MP	PLGA	6 months	Central Precocious Puberty	[247]
Vivitrol® Naltrexone	Vial with MP	75:25 PLGA	1 month	Alcohol & Opioid Dependence	[225]
Zilretta® Triamcinolone Acetonide	Vial with MP	75:25 PLGA	-	Osteoarthritis	[226]
Zoladex® Goserelin Acetate	Pre-filled syringe with implant	PLGA	28 days	Breast Cancer, Endometriosis, Prostate Cancer	[248]

Ref = reference; MP = microparticles; DCS = dual chamber syringe;

NMP = N-methyl-2-pyrrolidone

1.5.1 PLGA Based Formulations in Clinical Use

The polymer PLGA can be used to produce different types of biodegradable platforms. It is evident from table 6, that microparticulate based depot platforms are the most common ones used in clinics. The big advantage is the controllable size, which allows further regulation of drug release besides the choice of polymer. In general, smaller particles exhibit larger surface areas leading to accelerated hydration and rapid degradation [132]. Most microparticulate formulations are furthermore stored as lyophilized powders, exhibiting good physicochemical stability as opposed to liquid formulations. Carriers can be produced as nano-sized particles, offering further drug delivery strategies. Particles of this size show extravasation through blood vessels and tissues and can also be used as intracellular drug delivery vehicles. Especially after coupling with target ligands, selectivity for tissues or cells can be enhanced, presenting a promising approach for *e.g.* tumor targeting [56].

Another possible type of polymer based formulation is an in-situ forming implant, as demonstrated with AtriGel®. The aim of this technology was to combine simple and reliable implant devices with the convenient and easy administration of suspensions or solutions. Drug and polymer are thereby dissolved in a suitable and biocompatible water miscible solvent. Upon administration, the solvent diffuses out to nearby tissues and the polymer hardens due to the non-solubility in aqueous solutions. A solid implant is formed at the injection site, encapsulating the drug within for sustained-release [181]. While this system shows several advantages with respect to production, several problems can be expected for veterinary use from this type of formulation, since Buprenorphine SR-Lab® from ZooPharm described in chapter 1.3.1.3 uses a similar approach. Problems arose during administration of Buprenorphine SR-Lab® due to the high viscosity of the formulation. Furthermore, side effects at the injection site were a common phenomenon with the used solvent NMP, which is also utilized for AtriGel®. Also, stability of drugs and polymer can suffer from storage in liquid state [24,30,89,162,181,201].

Solid biodegradable implants are administered likewise to in-situ forming implants by subcutaneous or intramuscular injection. However, since a solid rod is implanted instead of a liquid formulation, application is more invasive and painful. For

comparison, injection of the microparticulate formulation Byduron® requires a 23 gauge needle with an outer diameter of 0.64 mm, while injection of implantable rods of Zoladex® requires a 16 gauge needle, resulting in more than doubled outer diameter of 1.65 mm [22,248]. Syringes delivering AtriGel® technology like Eligard® require needles with a gauge size of 18-20, which is still smaller compared to the solid implant technology [193]. Especially for small animals like mice and rats, needles should be kept as thin as possible.

1.5.2 Selection of a Suitable PLGA Type

The selection of a specific PLGA type for a novel formulation depends on many considerations, as hydrolytic degradation and thus drug release is affected by many factors. On the one hand, polymer properties like molecular weight, composition, hydrophilicity, morphology or glass transition temperature (Tg) impact formulation characteristics. On the other hand, additives like solvents and drugs can contribute likewise to change in degradation rate and release pattern [14,16,46,200].

PLGA is a co-polymer based on the monomers PLA and PGA as can be seen in figure 2. PLA exists in two optical isomers (D and L-lactide), showing a semi-crystalline structure. In contrast to PLA, crystalline PGA is lacking any methyl side groups as can be seen in figure 2, which results in higher hydrophilicity. Co-polymerization of L-PLA and PGA results in semi-crystalline polymers, while polymerization of D,L-PLA with PGA produces an amorphous structure. Polymers that consist of a higher ratio of D,L-PLA, are in general less hydrophilic, absorb less water and degrade for this reason slowly [132,240]. Additionally, higher PGA ratios (25-75%) reduce overall crystallinity of polymer and contribute to the increased rate of hydration and hydrolysis. However, degradation rate is not proportional to PGA content, as compositions of 50:50 PLA/PGA show a faster degradation compared to either homopolymer [132,143,149]. The ratio of PLA to PGA influences furthermore the glass transition temperature of the co-polymer. Below Tg, polymers transition from a highly viscous (glass) structure with rigid chain structure to a less viscous and more mobile form [15,132]. During transition,

diffusion coefficients of small molecules can increase drastically, influencing release kinetics greatly [107]. The transition temperature can be generally reduced by molecular weight reduction, reduction of PLA content and addition of plasticizing agents (*e.g.* solvents, drug, excipients) [2,14,16]. Molecular weight of used polymer is furthermore influencing degradation in general. Lower molecular weight PLGAs degrade faster, releasing drugs more rapidly [92,93,150]. Another customizable part of PLGA is the polymeric end-group. Terminal groups of polymeric chains can be either alkyl esters or acidic groups. Acid end groups are more hydrophilic and govern additionally autocatalytic degradation of PLGA, as they catalyze hydrolysis of polymeric ester bonds leading to the production of more acidic groups. Consequently, polymers with acidic end groups degrade faster [221,233]. Furthermore, interactions between polymer and drug or other excipients and solvents can influence formulation parameters profoundly. Encapsulated drugs, but also solvents like water can act as plasticizers, reducing Tg and increasing drug release [14,16]. Similarly, basic and acidic compounds can act as catalysts for hydrolysis of polymer, enhancing thereby degradation [46,200].

Selecting the right PLGA type is therefore depending on many factors and every new formulation has to be evaluated regarding intended use, release time, encapsulated compounds and preparation method.

1.5.3 Challenges During Development of PLGA Delivery Systems

Although controlled release formulations based on PLGA have been studied for several decades and the first formulation for humans was already introduced in the 1980s, only a few drug products managed to get market access [89,239]. Even though PLA and PLGA based drug delivery systems show many promising characteristics, several problems and technical barriers have been identified, impeding successful product development. Although degradation of PLGA to lactic acid and glycolic acid (figure 2) is considered as an advantage in terms of safety, the accumulation of both acids lead to a change of pH and can trigger an inflammatory response [5,63].

Furthermore, encapsulation of sensitive therapeutic agents like proteins becomes immensely complicated, as these compounds would not withstand harsh acidic environments [237]. Another repeatedly discussed challenge is the often-encountered high burst release of drug during the first hours after administration. Especially for depot formulations, which need to release their drug load over several months and have therefore a high total dose, a burst release of a few percent can already lead to high plasma concentrations causing systemic side effects [173]. However, a defined burst release can also be of advantage in terms of a loading dose [84]. A big challenge represents the interaction of the polymer with the entrapped drug. Small hydrophobic drugs may change formulation properties in this way by exhibiting plasticizing effects. For instance, studies showed that drugs like ketoprofen and 4-methoxy chalcone affected storage stability and manufacturing. To counteract plasticization and avoid physical interaction of drug and polymer, ketoprofen was included in β-cyclodextrin and 4-methoxy chalcone in hydrotalcite anionic clay [2,14]. Residual water shows a similar effect on PLGA formulations, as it lowers the glass transition temperature proportional to the residual amount [16]. Therefore, the storage environment of drug products based on biodegradable polymers needs to be water free. High residual moisture levels can furthermore lead to premature hydrolysis of polymer, altering drug release kinetics and stability [16,234,237].

Encapsulation of basic drugs can be also challenging, as they might accelerate PLGA degradation further by interaction with acidic chains. It was shown, that risperidone and olanzapine enhanced polymer degradation, as polymer weight was significantly reduced in loaded microspheres compared to un-loaded ones [46].

Control over drug release kinetics is a pre-requisite for successful development of drug delivery systems. Lack of reproducibility in respect to size and morphology of formulations can lead to variations in polymer degradation and drug release kinetics. Conventional methods like the often-used emulsification based microparticle preparation technique, may exhibit uncontrollable microparticle formation [21,118]. However, polydispersity does not have to be a disadvantage, as different sized particles may achieve tailor-made drug release. For instance, particle size of Risperdal Consta® is reported to be between 25-150 µm [182].

Production of PLGA based formulations requires non-polar solvents, which can dissolve high amounts of polymer but show low miscibility in water. Additionally, a high

vapor pressure is preferable to achieve removal of solvent by evaporation. For clinical application however, usage of solvents like dichloromethane, acetonitrile or ethyl acetate during production is a big concern. Elimination of these toxic solvents is a difficult task and residual amounts are highly regulated. Appropriate steps during production and analysis have to ensure low residual levels of used solvents [75,127,238].

Since most PLGA formulations are administered via parenteral route, sterility of these products has to be ensured. Typical sterilization procedures like dry or moist heat, sterile filtration or gas sterilization are not applicable due to the fragile nature of the polymer or the state of the formulation (*e.g.* it is not possible to sterile filtrate a microparticulate suspension). Therefore, sterilization is ensured either through aseptic processing or through terminal ionizing radiation [51]. However, it is often described, that the harsh conditions of irradiation alter polymer properties drastically. Thereby, changing drug release and impeding utilization of these techniques [1,23,76,128,208]. Moreover, regulatory barriers are high when it comes to PLGA based microparticles, as officially validated methods for dissolution and stability testing are not available. The lack of standardized in vitro and bioequivalence testing methods obstructs successful formulation development, which is evident by the absence of generic drug products [199].

Despite the challenges described here, PLGA based delivery platforms present essential features like biodegradability, formation of compatible metabolites and customizable drug release, making them a highly valuable tool for controlled drug delivery for a wide range of compounds.

1.6 Preparation Methods for PLGA Based Microparticles

Formulations based on microparticles are the most common ones for industrial production (table 6). Therefore, extensive studies have been conducted to characterize different preparation procedures and the resulting particles. Preparation methods for commercial use include mainly the solvent evaporation/extraction method, spray drying and coacervation [199].

1.6.1 Solvent Evaporation/Extraction Method

Many commercialized products are prepared by the solvent evaporation/extraction method [199]. Figure 3 illustrates the general preparation steps of this technique for single oil-in-water (O/W) emulsions and double (water-in-oil-in-water, W/O/W) emulsions. Encapsulation of hydrophobic drugs can be achieved by the single emulsion technique, as their solubility in the organic phase is high. In principle, drugs are either dissolved or dispersed in a polymer solution containing a volatile solvent (mostly dichloromethane) and emulsified in a next step in an aqueous phase containing tensioactive agents through agitation. Subsequent solvent removal is either done by evaporation or extraction [91]. Since PLGA is not soluble in water, partitioning of organic solvent leads to microparticle formation. For solvent evaporation, suspension is mildly agitated for several hours either at reduced or atmospheric pressure to allow complete fading of solvent. Using the extraction technique, the emulsion is transferred to a second water phase several times larger in volume, to allow complete diffusion of the solvent. However, for the evaporation technique, solvent needs to diffuse likewise to the water phase first to be able to evaporate afterwards. The difference is the speed of solvent removal, which has a big influence of particle morphology. Extraction results in rapid solvent removal and formation of porous particles in a more amorphous state [13].

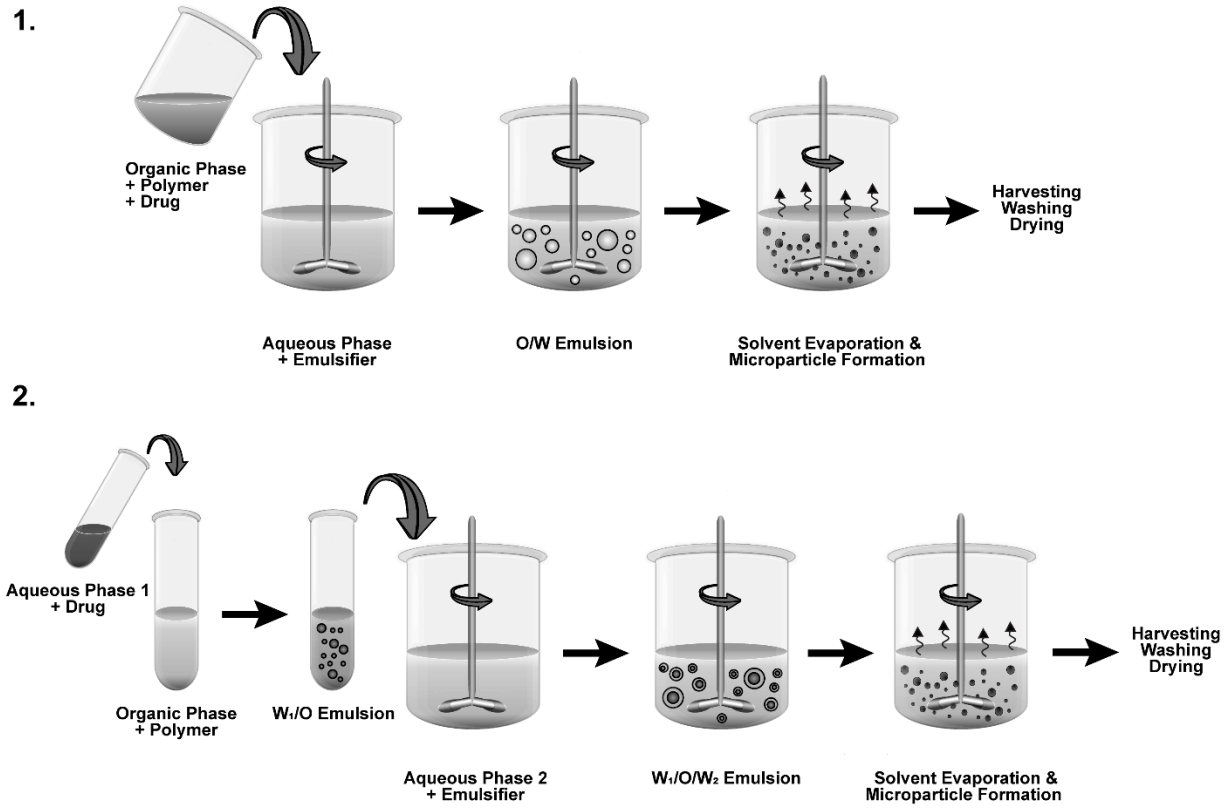

Figure 3: Major steps of microparticle preparation procedure by the solvent evaporation technique using either single emulsion (1) or double emulsion (2). For single emulsion technique (1), drug and polymer are dissolved in an organic phase. Subsequent emulsification of organic solution in aqueous phase is done through high-speed stirring. Residual organic solvent is removed by evaporation at either reduced or atmospheric pressure. Afterwards, microparticles are collected (e.g. centrifugation, filtration), washed and dried (e.g. lyophilization). For double emulsion technique (2), drug is initially dissolved or dispersed in a small aqueous phase and subsequently emulsified through intense shearing forces in the organic phase forming the first W/O emulsion. Afterwards, the first emulsion is added to a bigger second water phase and emulsified again to form the final W/O/W emulsion. Next steps are the same as described for the single emulsion technique.

Generally, parameters like pressure, temperature and the solubility of polymer in solvent and dispersion medium influence removal rate of solvent from suspension [91]. Subsequent recovery of spheres occurs through filtration or centrifugation. Particles are commonly washed to remove the non encapsulated drug and other excipients. Subsequent drying is commonly done by lyophilization to obtain free flowing powders. Hydrophilic drugs and sensitive compounds like proteins or peptides are prepared commonly by the double emulsion technique (figure 3) [91]. Using the O/W emulsion method for polar drugs leads to poor encapsulation efficiency, as partitioning of compound into the water phase during the emulsification process causes high losses. Drug is thereby deposited predominantly on the particle surface, leading to high burst release [13,15,91]. This can be prevented through introduction of an additional water phase. Polar drugs are hereby initially dissolved or dispersed in a small aqueous phase and subsequently emulsified under intense shearing forces, forming the first W/O emulsion. Afterwards, the first emulsion is added to a bigger second water phase and emulsified again under agitation to form the final W/O/W emulsion. Next steps are the same as described for the single emulsion technique. A large number of hydrophilic drugs, proteins and peptides have been successfully encapsulated by this method [49,50,91,110,134]. The solvent evaporation/extraction technique has been extensively studied not only in the laboratory but also in large-scale industrial production [199]. Technical effort and cost for this procedure are minimal, making it especially attractive for research. Furthermore, almost any compound can be encapsulated using the described method [15]. However, a severe obstacle is the low reproducibility and the limited control over particle size [199].

In the effort to overcome the drawbacks, an advanced microparticle preparation procedure based on membrane emulsification and solvent evaporation/extraction has been developed in the last decades [125,126,131,199]. A major difference between both techniques is the emulsification step of the dispersed phase in the continuous phase. In general, the dispersed phase, usually an organic phase or a premixed emulsion, is pushed through a membrane of given pore size into a continuous water phase. This passage through the pores produces homogenous droplets, leading to homogenous particles. Particle size is thereby influenced by the pore size and geometry, the shear flow applied on the continuous phase and on membrane

parameters like wettability, charge and permeability [199,227]. A promising membrane is Shirasu Porous Glass, which is successfully used to prepare uniform-sized microparticles [199,227]. Advantages of this method are the highly controllable particle size and narrow size distribution. This is achieved through homogenous pores and uniform energy input during particle formation. In contrast, conventional production using rotor-stator mixers to disperse the continuous phase, apply high shear forces to droplets close to the rotor, while droplets in other areas might encounter lower forces. This leads to polydispersity in particle size distribution. Additionally, emulsification through membranes generally applies low shear stress and operates under constant temperature, keeping sensitive compounds intact. However, disadvantages of this technique have been identified including low productivity and membrane fouling through interaction with solvents and compounds used [178,227]. Nevertheless, this technique shows potential for production of tailored uniform microspheres for various compounds [199]. Both, conventional and membrane emulsification solvent evaporation/extraction methods, proved to be valuable techniques for production, as can be seen on the approved PLGA formulations for clinical use.

1.6.2 Spray Drying

Spray drying is another technique used for industrial production of loaded PLGA microparticles [199]. Major steps of this preparation technique are presented in figure 4. In general, the drug is either dissolved or dispersed in an organic polymer solution. During the spray drying process, the liquid feed is atomized into small droplets using various types of nozzles. Individual droplets are further dried upon contact with inert gas (mostly nitrogen) and solid particles are formed. In a last step, dry particles are separated from the drying gas and collected [232]. Particle properties like size distribution and morphology are dependent on the design of the atomizer, the speed at liquid-gas interface during the atomization process, the viscosity of the bulk liquid, the solvent and the drying temperature. Through variation and monitoring of the mentioned parameters, particle size and density can be precisely controlled to achieve for example pulmonary delivery systems [95,133,232]. Furthermore, fast drying and

thereby transformation of micro droplets to solid particles contributes to the formation of amorphous solid dispersions. In this way, dissolution rates and bioavailability of poorly water soluble drugs can be increased significantly [61,176,232]. Production of microparticles through spry drying is a scalable one-step manufacturing technique, making it especially appealing for industrial production. Particles are thereby produced in a nearly continuous manner, which enables large batch sizes, high reproducibility and low levels of residual solvents and moisture [91,199,232]. Drawbacks of this technique are the high initial burst and the degradation of sensitive material. The initial burst stems from very fast evaporation of solvent during the drying procedure, which leads to migration of drug to the liquid-air interface and thus deposition on particle surface [199]. Degradation occurs on the one hand through elevated temperatures during drying and on the other hand due to the high shear stress during atomization [199,232].

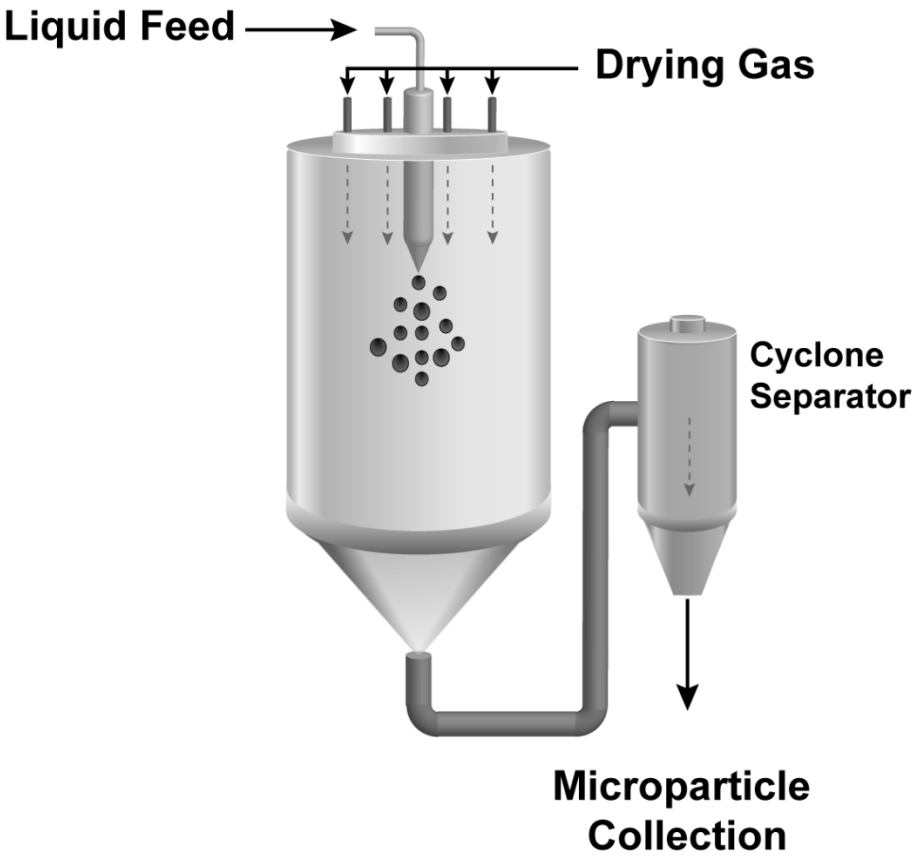

Figure 4. Major steps of microparticle preparation by spray drying. Liquid feed contains either dissolved, dispersed or emulsified drug in solution. Depending on nozzle (e.g. one-fluid, two-fluid) drug is either incorporated into the polymer solution or is fed as a separate solution. Likewise, atomization of liquid feed depends on nozzle (e.g. rotary, pressure). Particles are immediately dried through carrier gas (e.g. nitrogen) and collected in a cyclone.

1.6.3 Phase Separation by Non-Solvent Addition

PLGA based microparticles can be further produced by phase separation also called coacervation. This technique is based on the decrease of polymer solubility in an organic solution by addition of antisolvents, to enable drug coating [91,199]. The major steps of this process are (i) phase separation of the polymer solution, (ii) adsorption of the coating polymer around drug particles, and (iii) formation of solid microparticles as can be seen in figure 5. In general, the polymer is dissolved in a suitable organic solvent. Hydrophilic drugs (peptides, proteins) are dissolved in water and emulsified in the polymer solution forming a W/O emulsion. Hydrophobic drugs are directly added to the polymer solution and are either dissolved or dispersed. Addition of first antisolvent (*e.g.* silicone oil, vegetable oils, light liquid paraffin) leads to extraction of polymer solvent, phase separation and formation of coacervate droplets with encapsulated drug. Microparticles are completely hardened by addition of a second, large volume non-solvent phase (*e.g.* hexane, heptane) [91]. Subsequent steps (evaporation/extraction of solvent, harvesting, washing, drying) are the same as described for single emulsion evaporation/extraction technique in chapter 1.6.1. Antisolvent addition has to be slow to create a uniform coating of polymer around the drug. Furthermore, chosen non-solvents need to be miscible with the organic solution used for polymer solubilization but must not solubilize the active compound [199]. Beyond that, the second non-solvent should be volatile and extract efficiently the first non-solvent phase. Microparticle formation and consequently particle properties depend upon addition rate of non-solvents, stirring rate of polymer solution, ratio of aqueous phase for hydrophilic drugs to polymer solution, ratio of polymer solution to non-solvent solution and used drugs or excipients, as they might change the interfacial tension of dispersed phase against the organic phases [161]. A major drawback of this technique is the usage of different organic and potentially toxic solvents for polymer dissolution, coacervation and hardening of particles. Residual amounts of these agents have to be closely monitored and suitable extraction or washing steps have to be applied to ensure low solvent residues levels complying with regulatory requirements [217]. Another concern is the tendency of coacervate droplets to agglomerate. The absence of stabilizing agents leads to sticking of the soft droplets in the first phase

separation step. Thus, particle size and distribution cannot be precisely controlled [217].

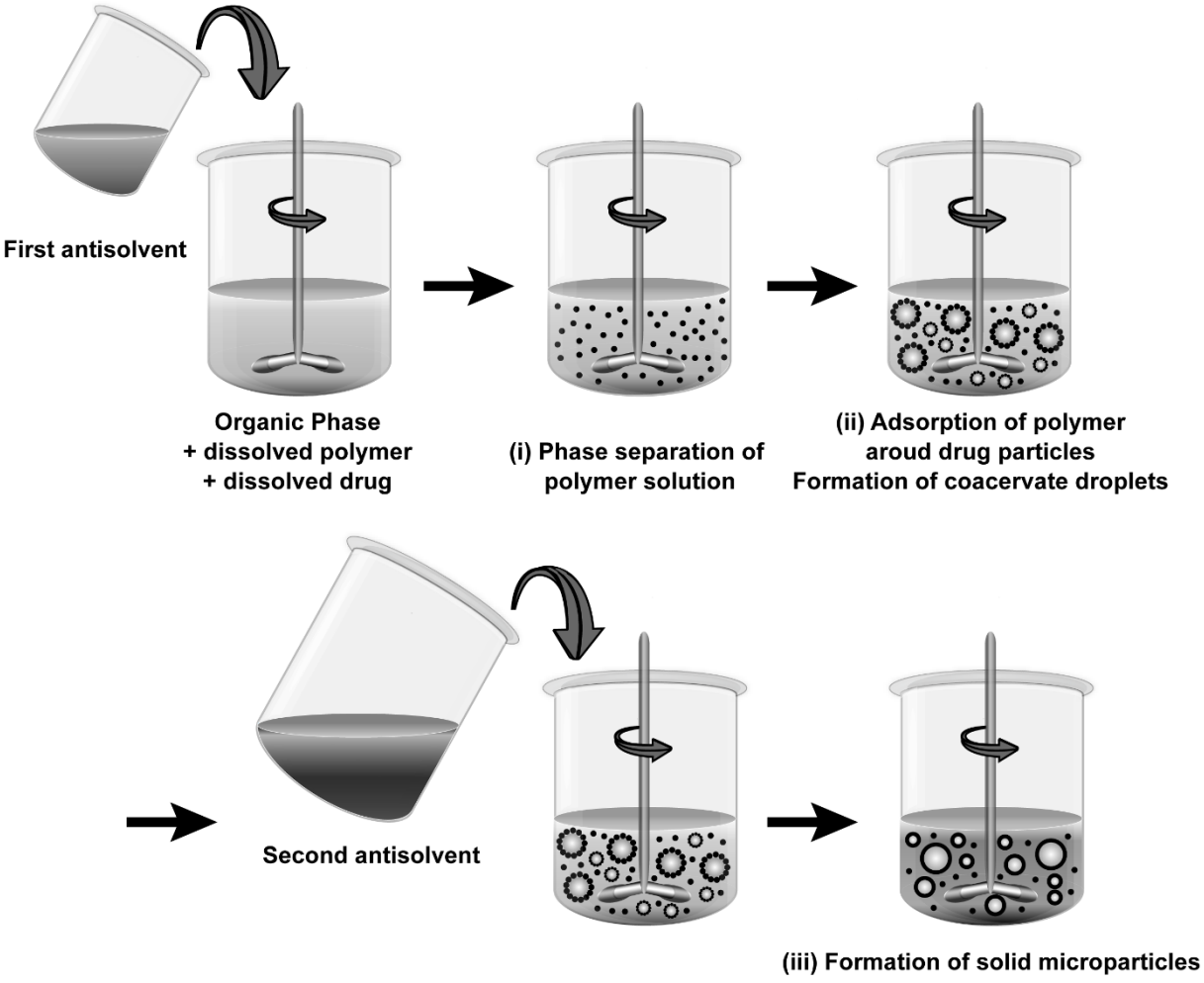

Figure 5. Major steps of microparticle formation using the coacervation method. Drug is either dissolved, dispersed or emulsified in the organic polymer solution. Addition of the first antisolvent leads to (i) phase separation of the polymer solution and (ii) adsorption of the polymer around drug particles. Thereby, soft coacervate droplets are formed. Addition of a second antisolvent phase leads to (iii) formation of solid microparticles. Subsequent steps are evaporation or extraction of solvent, harvesting, washing and drying of particles.

2. AIMS

Buprenorphine is a semisynthetic opioid derivative frequently used to ameliorate pain in animals after surgical interventions. However, due to its short terminal half-life, repeated injections are necessary to reach therapeutic plasma levels and analgesic effect over a prolonged period of time. Since no real alternatives for veterinary use exist until now to alleviate pain reliably after surgery, the aim of this project was to design and evaluate a safe and effective buprenorphine depot formulation to prolong analgesic effect in mice.

The goal was to develop a poly (lactic-*co*-glycolic acid) based microparticulate drug formulation with encapsulated buprenorphine hydrochloride for sustained parenteral drug release. Design of particles should include fast onset of action and a duration of analgesic effect for at least two days in mice. Furthermore, the formulation should be designed in a way to allow easy handling and simple injection volume adjustment. Additionally, pharmacokinetic and pharmacodynamic studies were conducted in mice to evaluate exposure of drug in plasma and brain tissue and to confirm prolonged pharmacological effect through analgesiometric assays. Moreover, this work should assess analgesic effect and identify any side effects of novel depot formulation in a surgical set-up. Thereby, sustained-release formulation should be compared to the current standard analgesic treatment protocols of non-retard buprenorphine formulations in rodents (Chapter I).

Additionally, the goal of this work was to assess manufacturing steps and characterize the novel depot formulation in terms of shelf life, use by period after reconstitution, residual moisture levels, sterility and endotoxin content for future industrial production and commercialization (Chapter II).

3. PUBLICATIONS

CHAPTER I

Design and In Vivo Evaluation of a Microparticulate Depot Formulation of Buprenorphine for Veterinary Use

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Personal contribution:
My contribution to this research article includes the design, planning and conducting of all experiments. Furthermore, I evaluated the results and prepared all figures and tables. I wrote the manuscript with the help of Jörg Huwyler, Paulin Jirkof and Pascal Detampel.

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Design and in vivo evaluation of a microparticulate depot formulation of buprenorphine for veterinary use

Viktoria Schreiner[1], Mattea Durst[2], Margarete Arras[2], Pascal Detampel[1], Paulin Jirkof[2,3]✉ & Jörg Huwyler[1]✉

Buprenorphine is a frequently used analgetic agent in veterinary medicine. A major drawback, however, is the short duration of action requiring several daily administrations. We therefore designed a poly-lactic-co-glycolic acid (PLGA) based microparticulate drug formulation for sustained parenteral drug release. Particles were designed to allow for a fast onset of action and a duration of the analgesic effect of at least two days in laboratory mice. Microparticles were produced using a solvent evaporation technique. Release rate was dependent on polymer type and particle size. Spherical particles used for subsequent animal studies had a mean size of 50 µm and contained 4.5% of buprenorphine. Drug release was characterized by an initial burst release of 30% followed by complete release over seven days. In vivo pharmacokinetic experiments in female C57BL/6 J mice confirmed prolonged exposure in plasma and brain tissue and correlated with the pharmacological effect in the hot plate assay or after minor abdominal surgery. No adverse side effects with respect to food and water intake, body weight, local tolerability, or nesting behavior were observed. Our formulation is an attractive alternative to established immediate release formulations. A use for prolonged pain management in laboratory animals is proposed.

Buprenorphine is a semisynthetic opioid derivative frequently used for laboratory and companion animals[1,2]. Displaying several favorable characteristics like a 25–40 times higher potency than morphine in mice and rats[3] and the occurring ceiling effect for respiratory depression[4–6], buprenorphine is commonly used to alleviate pain after surgical interventions in rodents[7]. However, due to its short half-life of around 3 h in rodents[8–10] repeated injections are necessary every 6 to 12 h[10,11]. The lack of available sustained-release buprenorphine formulations on the European market impede effective pain management and induce considerable stress on animals through the need of recurring injections and repeated animal handling[12]. Other opioids, which could be used as an alternative like morphine or fentanyl, fail to be as efficient as they are even more short acting[11,13,14]. Alternative routes of administration for buprenorphine, for instance the oral administration via drinking water, have also been discussed in recent years to reduce handling-associated stress. However, drinking behavior is dependent on the circadian rhythm[15,16], thus making it impossible to ensure adequate buprenorphine levels for every mouse and every time point post-surgery resulting in unreliable pain alleviation. Nevertheless, sufficient pain-relief is required for animal welfare and reduces the risk for stress-induced artefacts[17]. Therefore, there is an urgent need to develop a biocompatible sustained-release formulation to prolong analgesic effect in laboratory animals.

In the last decades the biodegradable and safe polymer poly (lactic-co-glycolic acid) (PLGA) has been extensively studied and various carriers with different preparation procedures for sustained drug release were invented[18–22]. The use in humans has already been approved by the FDA for various products demonstrating the suitability of this polymer for in vivo use[23]. Several different types and compositions of PLGA are commercially available, enabling a tailor-made release over days or even months[18,20]. It should be noted, that a sustained-release

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buprenorphine formulation based on a different delivery strategy was recently approved by the FDA for the use in humans[24].

Different sustained release products of buprenorphine appeared in the last years in the US for veterinary use. Buprenorphine SR-Lab® from ZooPharm (Fort Collins, CO) and Animalgesics for Mice® from Animalgesic Laboratories Inc. (Millersville, MD) are two FDA indexed formulations for experimental animals. Both formulations are depot formulations with a duration of action of at least 12–72 h in mice[25–27] and 48–72 h in rats[28–30]. They are legally marketed unapproved animal drugs for minor species. As such, they are not commercially available outside of the US. It was therefore the aim of the current study to provide an alternative for the European market. These existing products are based on in situ forming implants or liquid suspensions and have certain limitations. This includes lesions at the injection site such as erythema and necrosis, suboptimal injection volumes, and viscosity leading to handling problems[25,29,31–39]. The novelty of our approach consists thus in the use of a lyophilisate with a proposed extended shelf-life, a reduced risk for injection site reactions, a lower dose and thus a reduced risk for side effects as compared to Animalgesics for Mice®, and a lower viscosity as compared to Buprenorphine SR-Lab® allowing for convenient handling.

Here, a novel size-controlled microparticulate depot formulation of buprenorphine based on the biodegradable polymer PLGA for prolonged and controlled pain reduction is proposed. Several different polymers were tested and characterized regarding size, morphology, drug load and in vitro release to obtain a formulation with roughly 30% burst release and a sustained release up to three days. Subsequent pharmacokinetic studies in naive, adult, female C57BL/6 J mice were done to assess plasma and brain exposure. Analgesic action for the novel depot formulation was compared to a non-retard buprenorphine formulation (Temgesic®) with a thermal sensitivity assay and Mouse Grimace Scale (MGS) scoring after sham-ovariectomy. Side effects and impairment of mice after surgery were monitored by nest building behavior and by clinical parameters like body weight, food, and water intake.

Materials and methods

Materials. Buprenorphine HCl (BUP) EP/USP grade was purchased from Macfarlan Smith Limited (Edinburgh, United Kingdom). Buprenorphine and buprenorphine-D_4 (BUP-D4) standards in methanol (1 mg/mL) were obtained from Cerilliant (Round Rock, TX). Different types of PLGA 50:50 (Resomer RG 502, inherent viscosity (IV) = 0.20–0.22 dL/g), Resomer RG 502 H, IV = 0.20–0.22 dL/g, Resomer 503 H, IV = 0.38 dL/g) were purchased from Evonik (Essen, Germany). Polyvinylalcohol (PVA; M_w ≈67,000; 86.7–88.7 mol % hydrolysis), tris buffer and sucrose were obtained from Sigma Aldrich (St. Louis, MO). N,N-Dimethylformamide (analytical grade), dichloromethane (HPLC grade), methanol (HPLC grade), acetonitrile (MS grade), ammonium acetate (analytical grade) and sodiumlaurylsulfate (SDS) were obtained from Roth (Karlsruhe, Germany). HEPES was purchased from PanReac Apllichem (Darmstadt, Germany), sodium chloride (NaCl) analytical grade, formic acid and acetic acid (glacial) analytical grade from Merck (Darmstadt, Germany).

Preparation of buprenorphine loaded PLGA microparticles. Loaded microparticles were prepared by an oil-in-water (O/W) emulsion—solvent evaporation technique. Buprenorphine HCl (5 mg) and PLGA (100 mg) were dissolved in 5 mL of dichloromethane. The organic solution was then slowly injected over a time period of 7.2 min into 75 mL of ice-cooled 1% (w/v) PVA aqueous solution containing 1 M NaCl and 25 mM tris buffer with a pH of 9. Emulsification was done using a three-bladed propeller stirrer (RW 16 basic, IKA-Werke, Staufen, Germany) at 600 or 1200 rpm for 15 min. The resulting emulsion was transferred to a baffled flask and subjected to overnight agitation to remove residual organic solvent. Solid particles were collected through centrifugation for 10 min at 21′000 g and washed three times with 50 mL Milli Q water to remove PVA and not encapsulated drug. The resulting microparticle pellet was reconstituted in Milli Q water and distributed in vials. Sucrose was added (3.3 mg/mL) and vials were lyophilized for 24 h. The lyophilization process was initiated by lowering the shelf temperature to -45 °C over a period of 1 h (linear temperature gradient) followed by a sintering step (shelf at -45 °C during 3 h). Primary drying was done at 0.011 mbar during 17.5 h. Temperature was raised from -45 to 10 °C during that time (linear temperature gradient). Secondary drying was done at 30 °C during 3.5 h at 0.001 mbar.

Particle size and morphology of loaded microparticles. The size distribution of microparticles was measured by laser diffraction in water (MastersizerX, Malvern, Worcestershire, United Kingdom) using a lens suitable for particle sizes ranging from 1.2–600 µm. Particle size is expressed as the volume mean diameter and particle size distribution is expressed as 'span', which is defined by Eq. (1):

$$span = \frac{d(0.9) - d(0.1)}{d(0.5)}, \tag{1}$$

where d(0.1), d(0.5) and d(0.9) are the particle sizes at which 10%, 50% and 90% of the sample are below this size.

To analyze morphology, lyophilized particles were mounted on double-sided adhesive carbon tape that was fixed on an aluminum stud and sputtered with 20 nm gold (EM ACE600, Leica, Wetzlar, Germany). Surface and inner structure of loaded microspheres was examined using focused ion beam scanning electron microscopy (FIB-SEM; Helios NanoLab 650, FEI, Hillsboro, OR) as demonstrated previously[40]. Briefly, a focused gallium beam was used to remove the top layers of polymer as well as to prepare cross-sections of particles, which were subsequent visualized by SEM.

Measurement of drug loading and encapsulation efficiency. To measure drug content, lyophilized particles were dissolved in DMF, vortexed until a clear solution was obtained and analyzed by isocratic HPLC (Shimadzu Nexera X2 LC-30 AD, Kyoto, Japan). A reversed phase C_{18} column (Xbridge BEH, 4.6 mm × 50 m; 2.5 µm, Waters, Milford, MA) and a C_{18} guard column (SecurityGuard, 4.0 mm × 3.0 mm, Phenomenex, Torrance, CA) at 40 °C were utilized. A mobile phase of methanol:ammonium acetate buffer (20 g/L) :glacial acid (60:20:0.01) was delivered with a rate of 1.5 mL/min. BUP was detected at 288 nm. A stock solution of BUP in DMF was diluted with water to obtain a calibration curve ranging from 2 – 400 µg/mL.

Theoretical drug load (TDL) in percentage was calculated by Eq. (2):

$$TDL = \frac{m_{BUP}}{m_{BUP} + m_{PLGA}}, \tag{2}$$

where m_{BUP} is the total amount of BUP (mg) and m_{PLGA} is the total amount of PLGA (mg) used for this formulation. Actual drug load (ADL) in percentage was defined as the ratio between measured amount of drug in the sample and total mass of the sample. Encapsulation efficiency (EE) in percentage was calculated as ratio between ADL and TDL.

In vitro release study. To study the in vitro release, 60 mg of dried particles were dispersed in 7.5 mL 54 mM HEPES buffer containing 1.5% (w/v) SDS to ensure sink conditions. Samples of 300 µL for each time point in triplicate were incubated in closed glass vials at 37 °C on a horizontal shaker (250 rpm). To ensure homogenous mixing, vials were mounted on the shaker in a 45° tilted position. At specific time points, 260 µL of suspension was centrifuged for 8 min at 21′000 g and the supernatant was used for HPLC analysis to determine the cumulative release of BUP. Remaining particles were dissolved in DMF and analyzed by HPLC to obtain the total content of drug per vial in order to calculate normalized cumulative release. Burst release was defined as normalized BUP release after one hour.

Animals. 114 female 4 week old C57BL/6 J mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Housing conditions and experimental procedures were approved by the Cantonal Veterinary Office Zurich and Basel, Switzerland, under the license 30,583, and were in accordance with the Swiss Animal Protection Law and also conform to European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes and to the Guide for the Care and Use of Laboratory Animals[41].

A health surveillance program according to FELASA guidelines throughout the experiments monitored the animals' health status. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations[42].

Animals were housed in groups of 2–8 animals in Eurotype III cages or in groups of 2–6 in Eurotype II Long cages (Techniplast, Hohenpeissenberg, Germany) with autoclaved sawdust bedding (LTE E-001 Abedd, Indulab, Gams, Switzerland), one tissue paper and two nestlets (5 × 5 cm, Indulab), one cardboard hut (Ketchum Manufacturing, Brockville, Canada) and/or one red plastic house (Techniplast, Hohenpeissenberg, Germany), one wooden enrichment tool (40 × 16 × 10 mm, Abedd, Vienna, Austria) and a wooden platform (235 × 125 × 12 mm, Abedd). Animals had ad libitum access to food (3430.PX.S15 12 mm, Granovit, Kaiseraugst, Switzerland) and sterilized drinking water. Room temperature was 21 °C ± 1, humidity 45% ± 2 and a light/dark cycle of 12 h/12 h (lights on at 8 am) was implemented. Animals in experiments that contained surgical procedures, were single housed for a period of 5 days. All animals were tunnel handled. The animal room was insulated to prevent electronic and other noise. Disturbances, e.g., unrelated experimental procedures in the animal room, were not allowed. Animals were sacrificed for sample collection. In order to reduce the total number of animals used, some mice were reused for other experiments (table S1). Detailed information about age and weight of used mice for different experimental set ups can be found in the supplement (table S1-4).

Drug administration. Temgesic® (0.3 mg/mL buprenorphine HCl; Indivior Schweiz AG, Baar, Switzerland), a non-retard buprenorphine HCl formulation, was diluted with 0.9% NaCl (B.Braun, Hessen, Germany) to 0.02 mg/mL and given at a dose of 0.1 mg/kg body weight by subcutaneous injection in the lower belly region. Formulation RG 502 H-Big (BUP-Depot) was reconstituted with sterile saline solution to obtain a concentration of 240 µg/mL and injected subcutaneously with a dose of 1.2 mg/kg and a volume of 5 µl/g body weight once. Prior to injection, every batch of BUP-Depot was weighed and analyzed by HPLC regarding drug load.

Pharmacokinetic studies. Animals were housed in groups of 2–4 in Eurotype II Long cages as described earlier. All experiments started at 8:00 a.m. in the morning with a subcutaneous injection of either non-retard formulation or BUP-Depot. Mice were randomly allocated by group and sampling time point. Detailed information about weight of animals and injected doses can be found in supplement (table S2). At specific time points (0.5, 2, 5, 12, 24, 48, 72 h) animals were anesthetized using isoflurane (Attane, Lyssach b. Burgdorf, Switzerland). Anesthesia was induced with 5% Isoflurane, maintained at 3%, and the heart was punctured with a syringe. Blood was collected, transferred to EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Nürmbrecht, Germany), and centrifuged at 3′000 g, 4 °C for 10 min. 50 µL of plasma was transferred to a 96 well plate and stored at -20 °C until further analysis. Directly after blood sampling, the entire brain was carefully extracted and dissected using a scalpel or razor blade into the following tissues: cerebellum, medulla oblongata with pons and remaining brain (midbrain and forebrain). All parts were subsequently weighed and stored at -20 °C. The

specific binding of BUP in brain was calculated by subtracting drug concentration in cerebellum from combined concentrations in the remaining brain.

Sample preparation and liquid chromatogram- mass spectrometry analysis. Buprenorphine plasma and brain concentrations were determined by LC–MS-MS using a Shimadzu Nexera X2 LC-30 system (Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (Sciex QTRAP 6500, Framingham, MA). Deuterated buprenorphine (BUP-D4) was used as internal standard (IS). Calibration standards of buprenorphine covered a concentration range of 0.0032 to 50 ng/mL. Plasma (50 µl) and homogenized tissue samples were subjected to protein precipitation using acetonitrile, supernatants were collected, taken to dryness, and reconstituted with 100 µL of acetonitrile and 0.1% formic acid (4:6). For chromatographic analysis, two C_{18} columns were used (Sunshell 2.1 × 30 mm pre-column; 2.6 µm, ChromaNik Technologies, Osaka, Japan and Kinetex Biphenyl, 50 × 2 mm analytical column; 2.6 µm, Phenomenex, Torrance, CA). Gradient elution was used with dual-component mobile phase for both columns consisting of 0.1% (v/v) formic acid in water (Solvent A) and 0.1% (v/v) formic acid in acetonitrile (Solvent B) with a total flow rate of 0.45 mL/min. Column oven temperature was set to 60 °C and injection volume was 30 µL. Chromatographic separation was achieved by a gradient from 2% solvent B to 70% of solvent B over 6 min. Compound analysis was done in positive mode using a turbo spray temperature of 600 °C, an entrance potential of 10 V and an ion spray voltage of 4500 V. Detection of BUP and BUP-D4 was done using multiple reaction monitoring. Product ions with mass-to-charge ratios of 396.1, 414.2, 152.0, 165.1 were monitored for BUP and 400.1 (m/z) for BUP-D4.

Pharmacodynamic studies. *Analgesiometric assay.* Animals were housed in Eurotype II long cages during the experiments and housing conditions of mice were the same as described above. Detailed information about weight of animals and injected doses can be found in supplement (table S3). At 8:00 a.m., equal numbers of mice were randomly assigned to receive subcutaneously, either one injection of BUP-Depot, non-retard formulation or saline solution with similar volume. The investigator performing the analgesiometric assay was blinded to treatment. Mice from each experimental group were tested at specific time points post injection (2, 12 or 24 h). Mice that received BUP-Depot were additionally tested 48 h post injection. Analgesic action was assessed by the hot-plate method[25,43]. Briefly, mice were habituated to the test room for 10 min and placed afterwards on a 54 ± 1 °C hot plate. Time was measured until mice showed one of the following behaviors: Hind-paw lick, hind-paw shake or jump. All experiments were either recorded by camera from two opposite sides of the hot plate or just from one side, while a mirror was placed on the other side to help detect the aforementioned behaviors. The investigator was watching the animals from a third side to remove animals from the plate after signs of nociception or a cut off time of 40 s. All latency measurements and evaluations were done blinded based on recorded video material.

Assessment of pain relief and side effects after minor surgery. For the surgical procedure, 12 mice aged 14 weeks were housed individually for the experiment in Eurotype III cages with pre-weighed water and food. Detailed information about weight of animals and injected doses can be found in supplement (table S4). One fresh nestlet (5 cm × 5 cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland) was given to each mouse to observe their nest building behavior before and after surgery. Two-times four animals per experiment were used, which were randomized to one of the two experimental groups: One injection BUP-Depot or two injections non-retard formulation. Experimental procedure was as follows:

Day 1: At 8 a.m. individual housing started with a new nestlet and pre-weighed food and water. Animals were weighed for baseline measurement.
Day 2: At 8 a.m. assessment of nest complexity score, monitoring of body weight, food and water intake was done. Baseline measurements for the Mouse Grimace Scale were conducted at 12 a.m., 2 p.m. and 9 p.m.
Day 3: Prior to surgery at 8 a.m. assessment of nest complexity score, body weight, food and water intake was done.

Surgery. Both groups received at 8:00 a.m. either non-retard formulation or BUP-Depot. Animals were transferred to the surgery room. At 9 a.m. anesthesia was induced via nose mask (5% isoflurane, 600 mL/min gas flow), animals were transferred to a warmed (39 ± 1 °C) operating table, and anesthesia was maintained (3% isoflurane, 600 mL/min gas flow) via nose mask. Eye ointment was applied, the fur was clipped and removed and the surgical site was disinfected with Braunol (B. Braun Medical AG, Sempach, Schweiz). Mice underwent a one-side sham embryo transfer as described previously[44]. Surgery was completed within 3–4 min in the surgery groups and total anesthesia time was 10 ± 1 min. While regaining consciousness after anesthesia, animals stayed for ~10 min on the warmed table and were transferred afterwards to a warming cabinet (32 °C) for 60 min before returning to the housing room.

Mouse Grimace Scale measurements were done 3, 5- and 12 h post-surgery. Six hours post-surgery animals in the non-retard formulation group received their second injection with the same dose.

Day 4: At 8 a.m., monitoring of nest complexity score, body weight, food and water intake after surgery.
Day 5: At 8 a.m., monitoring of body weight, food and water intake after surgery.

Formulation	Mw (kDa)	Endgroup	Stirring Speed (rpm)	Size (µm)	Span	Actual Drug Load (%)	Encapsulation Efficiency (%)	Burst Release (%)
RG 502 H-Small	13.4–15.1	–COOH	1200	11.7 ± 2.5	1.8 ± 0.1	3.5 ± 0.1	72.9 ± 2.0	15.7 ± 5.1
RG 502 H-Big* (BUP-Depot)	13.4–15.1	–COOH	600	49.8 ± 17.4	2.5 ± 0.4	4.5 ± 0.8	94.5 ± 12.9	28.1 ± 5.2
RG 503 H-Small	29.4	–COOH	1200	14.7 ± 4.2	1.7 ± 0.4	3.5 ± 0.1	72.7 ± 1.9	5.9 ± 2.4
RG 503 H-Big	29.4	–COOH	600	34.5 ± 10.3	1.5 ± 0.4	3.8 ± 0.5	82.1 ± 11.2	27.7 ± 10.6
RG 502-Big	13.3–13.4	–COOR	600	51.3 ± 22.5	2.6 ± 1.5	3.4 ± 0.3	71.4 ± 6.1	8.4 ± 5.8

Table 1. Characteristics of sustained-release buprenorphine loaded microparticles. Data is presented as mean ± SD (n ≥ 3). *This formulation is the lead formulation used for all subsequent animal experiments. It is referred to as BUP-Depot.

Nest building behavior, body weight, food and water intake. Nest complexity score was determined every morning at 8:00 a.m. prior to any intervention by a blinded observer using a scoring system (Score 0 -5) as described previously[44].

Assessment of overall welfare was done through monitoring of body weight, food and water intake. Body weight of all animals as well as weight of food and water was measured every morning prior to any intervention.

Mouse grimace scale. One day prior to surgery, baseline measurements of Mouse Grimace Scale were done as previously described[45] at 12 a.m., 2 p.m., and 9 p.m. (corresponding to 3, 5 ,and 12 h after surgery). Briefly, mice were placed in Plexiglas cubicles (9 × 5 × 5 cm high) and filmed for 5 min. Frontal pictures of animals were generated and five were chosen for each mouse and time point and scored independently by two blinded observers for signs of pain. Pain scores were averaged for both observers. After surgery, the procedure was repeated at the same time points. Out of the five possible action units to code (orbital tightening, nose bulge, cheek bulge, ear position and whisker change), whisker change had to be excluded from analysis, as animals after surgery tended to stain the Plexiglas with eye ointment, making it difficult to examine the whiskers of mice properly. Values of individual animals were only included in results if three or more pictures could be completely scored. Delta Mouse Grimace Scale scores were obtained by subtracting the mean for the "after-surgery photographs" from the mean for the "baseline photographs" for each mouse and time point individually.

Local-effects on injection site. Post injection of BUP-Depot and non-retard formulation, all mice were observed for local effects on injection site. If abnormalities like redness or swelling were detected, skin around injection site was removed post mortem and histologically analyzed.

Statistical analysis. All statistics were done using OriginPro 2018 software (OriginLab Ltd, MA). Sample size calculation was based on previous experiments[43] using the software GPower 3.1[46]. All data were tested for normality by performing a Shapiro–Wilk test. Analysis of variance (ANOVA) and two tailed t-tests were used. Level of significance was $P < 0.05$. For hotplate assay, ANOVA was combined with Tukey post hoc test. Baseline data for body weight, food and water intake were obtained by averaging measurements done on day 1 and 2 of experimental procedure. Scores were analyzed regarding significant differences between BUP-Depot and non-retard formulation group using non-parametric Mann–Whitney test.

Results

Characterization of buprenorphine loaded microparticles. Size, ADL, EE and burst release of different PLGA microparticle formulations are listed in Table 1. Burst release was defined as drug release after one hour and can be attributed to BUP encapsulated on or close to surface, easily accessible for hydration. The results showed that stirring speed had a major influence on particle size. Doubling the stirring speed with the polymer RG 502 H from 600 to 1200 rpm resulted in a decreased size of 11.7 µm compared to 49.8 µm, respectively. The same tendency could be seen with the higher molecular weight polymer RG 503 H, where particles produced with a higher stirring speed were only half the size of the particles produced with 600 rpm, namely 14.7 µm compared to 34.5 µm. Furthermore, particles with different sizes and same type of polymer showed differences regarding ADL, EE and burst release. Bigger particles of the polymer RG 502 resulted in an overall higher drug load (4.5% vs. 3.5%) and a higher EE (94.5% vs. 72.9%). Additionally, the larger particles displayed almost a doubling of the burst release (28.1%) compared to the smaller ones (15.7%). The same trend could be detected for the formulations prepared with RG 503 H. RG 502 H and RG 503 H have different molecular weights but very similar inherent viscosities of 0.22 dl/g and 0.38 dl/g. Particles have therefore similar size, ADL, and EE. However, burst release was slightly lower for smaller RG 503 H particles. Noticeable differences could be observed for particles formulated with the polymers RG 502 and RG 502 H. Both polymers consisted of similar chain lengths but differed regarding end capping. Particles formulated with these capped polymers using the same stirring speed of 600 rpm were only comparable in respect of size, but in terms of drug load, EE, and burst release they resembled more formulations with smaller particle sizes of the uncapped polymers (RG 502 H-Small or RG 503 H-Small). Here, ADL, and EE were almost identical, as well as burst release with 15.7% for RG 502 H-Small and 8.4% for RG 502-Big.

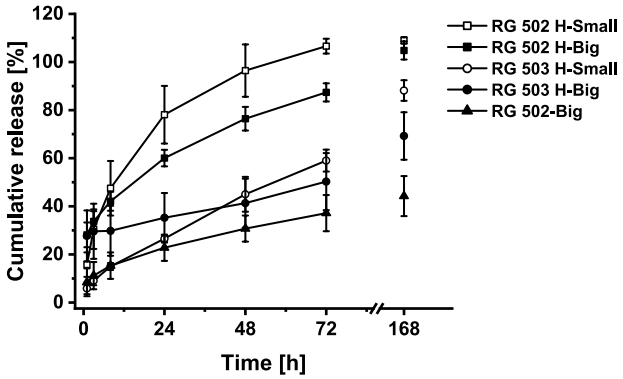

Figure 1. Effect of particle size and type of polymer on in vitro release of buprenorphine HCl loaded PLGA microspheres. Release was studied in 54 mM HEPES buffer at pH 7.4 with 1.5% SDS at 37 °C. Data is presented as mean ± SD (n ≥ 3).

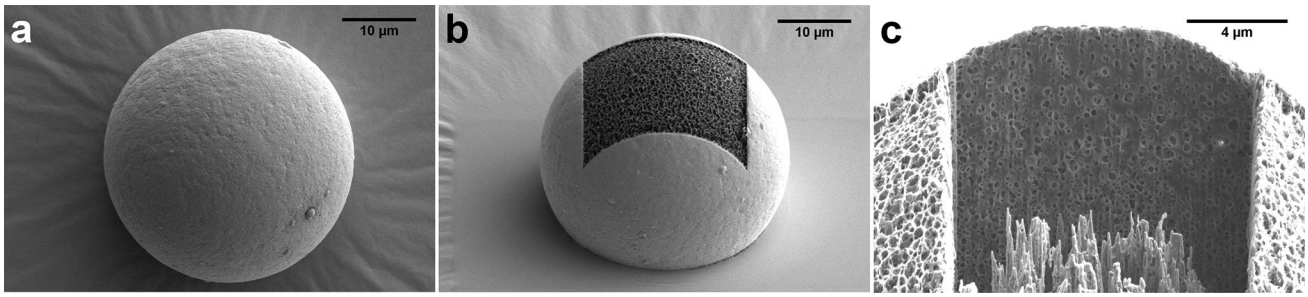

Figure 2. Scanning electron microscopy pictures of buprenorphine loaded PLGA (RG 502 H) microspheres**.** Focused ion beam technique was used to examine inner structure through removal of top layer of polymer (**b**) and vertical sectioning of particles (**c**).

Effect of particle size and type of polymer on in vitro dissolution. In vitro release profiles of drug loaded microparticles in HEPES buffer (pH 7.4) incubated at 37 °C are shown in Fig. 1. Particle size and the type of polymer affected the in vitro release of buprenorphine from PLGA particles. Smaller particles in general displayed a faster release as could be seen in formulation RG 502 H-Small compared with RG 502 H-Big. Here, smaller particles reached 100% release after 72 h while the bigger particles attained only 87%, even though they had a higher burst release. RG 502 H-Big reached complete release between 3 and 7 days. A comparison of big and small particles prepared with the polymer RG 503 H showed the same trend. Hence, big and small particles reached almost the same release after 72 h of 50–59%, but the initial 20% higher burst release of the bigger particles meant that the release rate was much lower. Formulations with RG 503 H did not reach complete release within 7 days, but retained approximately 20–30% of drug. Release characteristics differed between polymers with higher and lower molecular weight. As expected, longer polymer chains demonstrated a slower release of the drug, as could be observed for RG 502 H-Small and RG 503 H-Small. Both formulations presented a similar particle size, but after 72 h only half (59%) of buprenorphine was being released from the higher molecular weight polymer compared to a complete release from the lower molecular weight version. Additionally, formulations with RG 503 H presented an almost zero order release after initial burst phase for the duration of 72 h. In contrast, the release profile of formulations with RG 502 H showed a power law release kinetic. This study showed furthermore that acidic end groups of polymers like RG 502 H and RG 503 H had an increasing effect on BUP release in vitro. Particles prepared with RG 502 H resulted in a complete drug release after 3 days, while particles with the same size formulated with capped polymer RG 502 released only 37%. Formulations with this type of polymer followed a power law release kinetic similar to RG 502 H. Formulation RG 502 H-Big, with a complete drug release of 3 days combined with a burst release of around 30%, was further characterized and used for all subsequent in vivo studies.

Morphology and inner structure of loaded particles. Microparticle morphology was studied using scanning electron microscopy (Fig. 2a). BUP loaded particles formulated with the polymer RG 502 H showed a uniform, spherical shape and a smooth outer surface. In contrast, the inner structure of the spheres, revealed through ablation by a focused ion beam, was highly porous (Fig. 2b, c). Pores were homogeneously distributed throughout particles as can be seen from the vertical sectioning image in Fig. 2c.

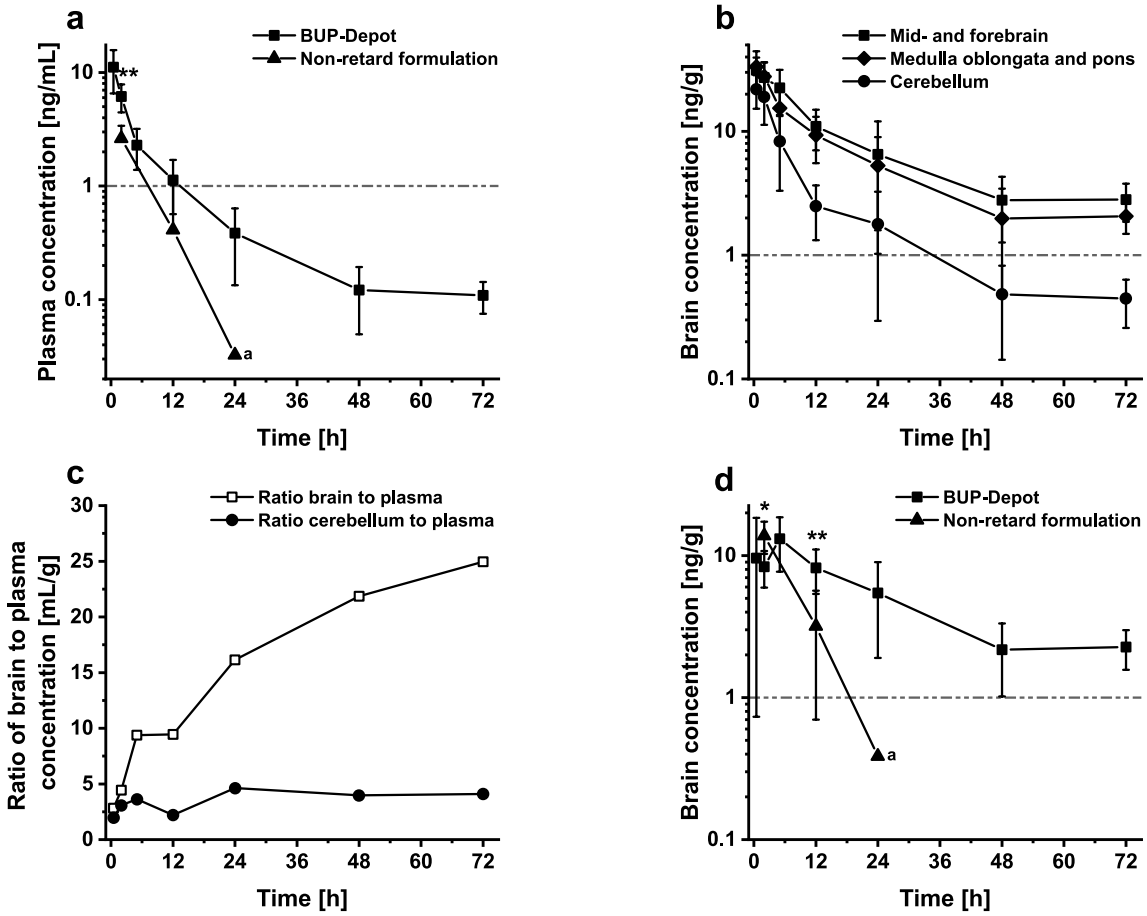

Figure 3. Plasma and regional brain concentration–time profiles. (**a**) Plasma concentration–time profile of RG 502 H-Big (BUP-Depot) and non-retard formulation. Unpaired t-test showed significant difference between formulations for 2 h ($P = 0.0009$) but not for 12 h ($P = 0.051$). (**b**) Regional brain concentrations of BUP-Depot in mid-and forebrain, medulla oblongata and pons, and cerebellum. (**c**) Brain-to-plasma ratio-time profiles of BUP-Depot. Brain concentrations are defined as sum of concentrations of mid- and forebrain, medulla oblongata and pons. (**d**) Specific binding of BUP-Depot and non-retard formulation in brain. Specific binding is defined as difference between combined concentrations of mid- and forebrain, medulla oblongata and pons and concentrations in cerebellum. Unpaired t-test showed significant difference at 2 h ($P = 0.01$) and 12 h post injection ($P = 0.006$) between both formulations. BUP-Depot (1.2 mg/kg) and non-retard formulation (0.1 mg/kg) were injected once subcutaneously. Dashed line in all graphs represents threshold of 1 ng/mL or 1 ng/g. Data expressed as mean ± SD. a: 24 h time point of non-retard formulation could not be statistically analyzed as only 2 out of 7 animals showed values higher than LLOQ for concentrations in plasma and cerebellum. Values below LLOQ were excluded, therefore no SD value is shown. ($P < 0.05$ *; $P < 0.01$ **).

Pharmacokinetics. Analytes were measured by LC–MS-MS in positive mode with a LLOQ of 0.008 ng/mL for plasma and 0.048 ng/mL for brain tissue. Single injection of BUP-Depot led to a biphasic release profile in mouse plasma as indicated in Fig. 3a. The first part of the profile could be attributed to the burst release of the retard formulation until 5 h post injection. Following the initial fast and immediate release of BUP, a slower sustained release phase could be observed until 72 h. At 12 h post injection, plasma concentrations started to approximate the mark of 1 ng/mL with a concentration of 1.1 ng/mL. Thereafter, plasma levels decreased further until a value of roughly 0.1 ng/mL was reached for the duration of 72 h. In contrast, a single injection of non-retard BUP formulation presented a profile with only one phase, where plasma concentrations above 1 ng/mL could only be measured up to two hours post injection. After 12 h plasma concentration dropped to 0.4 ng/mL but no significant difference to BUP-Depot could be established ($t(11) = 2.20$, $P = 0.051$). At 24 h post injection only 2 out of 7 mice showed detectable drug concentrations, whereas all other values were below the limit of quantification and were not included. Therefore, no statistical analysis could be performed.

In addition to plasma, brain tissue as the target site of BUP was analyzed. Figure 3b shows BUP concentrations in different parts of mouse brain after a single injection of BUP-Depot. In both tissue fractions, (medulla oblongata with pons and mid-/forebrain) concentrations above 1 ng/g for the whole duration of 72 h were observed. While medulla oblongata and pons and mid-and forebrain displayed similar concentrations of drug over 3 days, the amount of BUP in the cerebellum was much lower. Same trend can be seen in mice receiving non-retard

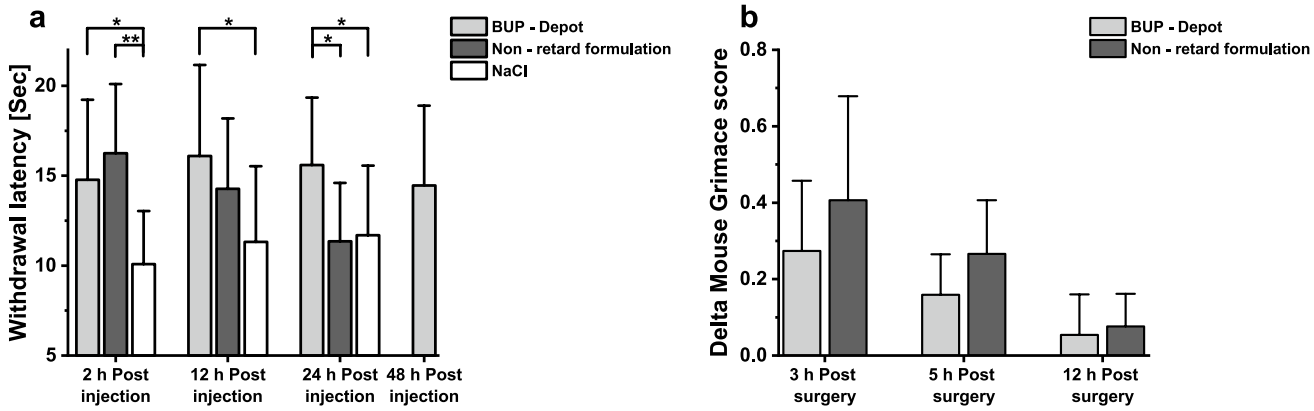

Figure 4. Effect of sustained-release buprenorphine (BUP-Depot), non-retard formulation and saline on withdrawal response latency to thermal stimulus of 54 °C in mice and on delta mouse grimace score after sham-ovariectomy. (**a**) One way analysis of variance with post hoc Tukey test shows significant (*$P<0.05$;**$P<0.01$) increase in latency for BUP-Depot at 2 h, 12 h, 24 h time points compared to NaCl and compared to non-retard formulation at 24 h. Non-retard formulation shows only a significant increase in latency at the two hours' time point compared to NaCl animals. ($n \geq 11$ per time point and group). (**b**) Standard analgesic protocol was used after sham-ovariectomy, treatment groups received either one injection of BUP-Depot or two injections of non-retard formulation 6 h apart. No significant difference between delta mouse grimace scores could be shown for both groups 3–12 h post-surgery using Mann–Whitney test ($\alpha = 0.05$). Data expressed as mean ± SD for all experiments. ($n = 6$ per group).

formulation (Supplement, figure S1). This difference could be attributed to differential expression of opioid receptors in the brain, i.e. absence of receptors in cerebellum which was therefore used as baseline control tissue[47].

Consequently, the ratio of cerebellum to plasma concentrations represents unspecific perfusion and does not change. In contrast, brain (i.e. mid-/forebrain combined with medulla oblongata and pons) to plasma concentrations increased over time by a factor of eight being indicative of specific receptor binding and tissue accumulation (Fig. 3c).

Based on considerations above, 'specific binding' of drug in the brain was defined as difference between combined concentrations of mid- and forebrain, medulla oblongata and pons and concentrations in cerebellum (Fig. 3d). In all subsequent experiments, the term 'specific binding' refers to this differential value. The maximum reached specific binding concentration of BUP was similar for the non-retarded and the depot formulation, 13.8 ng/g and 13.2 ng/g, respectively. However, the time until the maximum concentration of drug was reached was increased for the BUP-Depot formulation with 5 h compared to the non-retard formulation with 2 h. At 12 h post injection concentrations achieved through depot formulation were significantly higher compared to non-retard solution ($P = 0.006$). At 24 h post injection the specific binding concentration for BUP-Depot was still above 1 ng/g with 5.4 ng/g, whereas mice receiving the non-retarded formulation experienced a much lower value, 0.4 ng/g. Here the concentration of BUP in cerebellum could not be detected for 5 out of 7 mice, as concentrations dropped below LLOQ and were not included in the analysis. In contrast, up until the last measurement at 72 h post injection BUP-Depot still demonstrated concentrations above 1 ng/g with a value of 2.3 ng/mL. Only one mouse showed drug concentrations below LLOQ.

Pharmacodynamics. *Analgesic efficacy of BUP-Depot compared to non-retard formulation.* Withdrawal latencies in response to heat stimulus of 54 ± 1 °C were measured after a single injection of either BUP-Depot, non-retarded BUP, or NaCl. Results of hotplate assay are presented in Fig. 4a. First testing was performed two hours post injection, as non-retarded BUP should display a maximum effect at that time, which served as a positive control. Further experiments were conducted 12 and 24 h post injection for all three treatment groups and 48 h only for BUP-Depot, since it was the only group where a pain reduction was still conceivable. Analysis of variance followed by post hoc pairwise comparison of means with Tukey correction revealed that 2 h post injection withdrawal latencies were significantly higher for BUP-Depot ($P = 0.013$) and non-retard formulation ($P = 0.001$) compared to control group which received NaCl. Furthermore, 12 h post injection mice receiving sustained-release BUP displayed significantly higher latencies compared to NaCl control animals ($P = 0.03$). No significant difference was found between latencies for non-retard formulation compared to NaCl ($P = 0.24$), confirming the short duration of action of the commercial formulation. Tested again 24 h post injection, withdrawal latencies for BUP-Depot were still significantly higher compared to baseline ($P = 0.038$) and to non-retard formulation ($P = 0.02$). Mice receiving commercial non-retard formulation displayed comparable latencies as NaCl animals ($P = 0.97$), which is in line with the pharmacokinetic data which showed that 24 h post injection BUP concentrations in plasma or brain were very low. The latest analgesiometric test was performed 48 h post injection only with a BUP-Depot group as latencies of control animals receiving NaCl did not vary much between different time points and no effect of non-retard formulation could be expected. Withdrawal latencies for animals receiving BUP-Depot were still higher at 48 h post injection with 14.5 s compared to baseline

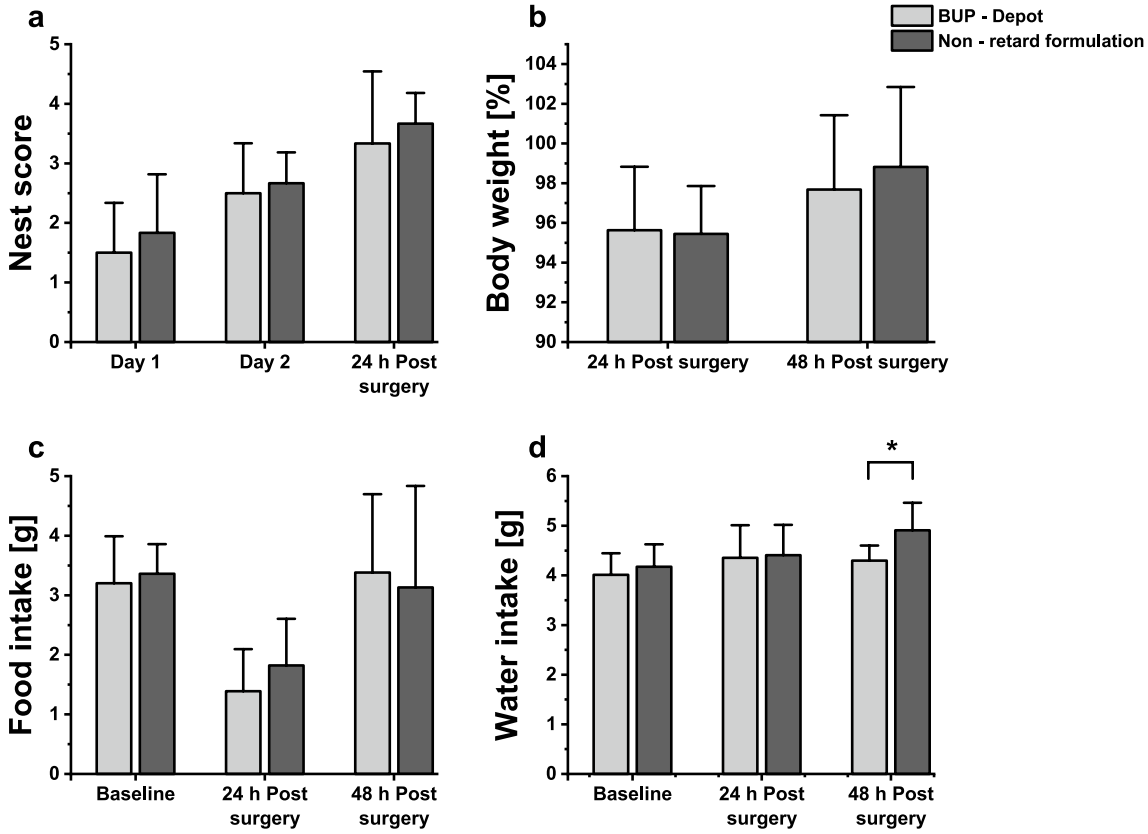

Figure 5. Effects of one injection of BUP-Depot compared to two injections of non-retard formulation after sham-ovariectomy on nest complexity score, body weight, food and water intake. (**a**) Nest scores during baseline measurements on day 1 and 2 and 24 h post-surgery show no significant differences between both groups at any time point (Day 1: $P = 0.92$; Day 2: $P = 0.73$; 24 h: $P = 0.92$). (**b**) Body weight change in percent 24 and 48 h post-surgery show no significant difference between BUP-Depot and non-retard formulation using unpaired two-tailed Student t-test (24 h: $P = 0.91$; 48 h: $P = 0.62$). Food intake (**c**) and water intake (**d**) in gram during baseline measurements, 24, and 48 h post-surgery. Baseline measurements consists of average data from day 1 and day 2 of procedure. No significant difference between non-retard formulation and BUP-Depot group could be found using unpaired two-tailed Student t-test ((**c**) Baseline: $P = 0.69$; 24 h: $P = 0.34$; 48 h: $P = 0.78$), (**d**) Baseline: $P = 0.54$; 24 h: $P = 0.88$). Only exception was 48 h post-surgery water intake, indicating significant difference between BUP-Depot and non-retard formulation group ($P = 0.038$). Data is expressed as mean ± SD. (n = 6 per group).

animals measured 24 h post injection with 11.7 s, but unpaired t-test showed that difference was not significant ($t(21) = 1.59$, $P = 0.13$).

Analgesic action of BUP-Depot compared to non-retard formulation was further assessed after surgery with standard analgesic protocol to alleviate pain. Mouse Grimace Scale was used to determine pain of animals 3, 5 and 12 h after surgery (Fig. 4b). Although a tendency towards lower delta MGS scores indicating lower pain was seen at 3 and 5 h, no significant difference between treatment groups for all time points could be shown (3 h: $U = 11.5$, $P = 0.34$; 5 h: $U = 9$, $P = 0.17$; 12 h: $U = 19$; $P = 0.94$).

Correlation between drug concentration in plasma or brain and analgesic effect. Figure 3a and 3d present drug concentrations of BUP-Depot in plasma as well as in the brain. Both profiles showed a different distribution of buprenorphine over the course of 72 h. While plasma concentrations fell below 1 ng/mL after 24 h, the drug concentration in the brain remained above 1 ng/g for the whole duration of 72 h. Combined with the analgesic effect shown in Fig. 4a, a better correlation between drug-brain-concentrations and analgesic action could be shown compared to the plasma concentration.

Effect of BUP-Depot on nest building behavior, body weight and food and water intake after surgery compared to non-retard formulation. The goal was to show similar effect of non-retard and sustained release formulation on mice after surgical procedure. Therefore, no difference was expected between both groups. Reduced nest building behavior is used as indicator of distress after surgical interventions in mice[44,48]. Analysis of nest scores of animals receiving either one injection BUP-Depot or two injections of non-retard formulation after sham-ovariectomy are presented in Fig. 5a. Regardless of the received formulation, mice built and improved their nests. Both groups had similar scores of 3.3 and 3.7 for BUP-Depot and non-retard

BUP treated animals 24 h post-surgery, respectively. No significant difference could be found 24 h post-surgery between both groups (U = 17, $P = 0.92$). Body weight change (Fig. 5b) was not significantly different between non-retard formulation and BUP-Depot group for all time points (24 h: t(10) = 0.12, $P = 0.91$; 48 h: t(10) = -0.51, $P = 0.62$). Mice lost around 4% of their body weight after surgical intervention 24 h post-surgery. Already 48 h after surgical intervention, body weights of both groups (97.7% for BUP-Depot and 98.8% for non-retard solution) were similar compared to baseline, hinting that both groups recovered fast. Similar to body weight change, food intake was not significantly different if Depot and non-retard group were compared before or after surgery (Fig. 5c) (Baseline: t(10) = -0.41, $P = 0.69$; 24 h: t(10) = -1.01, $P = 0.34$, 48 h: t(10) = 0.28, $P = 0.78$). Mice ate less 24 h post-surgery, around 1.4 g of pellets for BUP-Depot and 1.8 g for non-retard formulation. Food intake for both groups was again similar to baseline values 48 h after the surgical procedure, indicating good recovery. While no significant difference could be found 24 h post-surgery between water intake for mice that received BUP-Depot or non-retard BUP (t(10) = -0.15, $P = 0.88$), after 48 h post-surgery animals that received two injections of non-retard formulation showed a significant increase in water intake (Fig. 5d, t(10) = -2.38, $P = 0.038$).

Local-effects on injection site. Only two mice out of 75 receiving the BUP-Depot formulation demonstrated a local reaction after 48 h, which manifested as mild skin irritation and swelling. Post-mortem analyses revealed one animal with a mild inflammation and another animal with a focal area of necrosis at injection site (data not shown).

Discussion

Animal welfare is of paramount importance in biomedical research and includes the critical analysis of existing pain management protocols. One of the most commonly used analgesics to relieve moderate to severe pain in rodents is the opioid buprenorphine. However, its duration of action of only 6 -12 h requires repeated injections after most surgical interventions[11].

In the present study, the biodegradable and biocompatible polymer poly (lactic-co-glycolic acid) was chosen to achieve prolonged drug release. PLGA is typically utilized to attain sustained-release over the course of several weeks (i.e. Lupron Depot, Eligard, Nutropin Depot and Profact). Buprenorphine is commonly administered for post-surgical pain after moderate to major surgeries for up to three days. Hence, ratios of 50:50 polylactic acid to polyglycolic acid were used in this study, as this composition is described to be fast releasing compared to bigger shares of PLA[49,50]. Furthermore, the molecular weight of the elected polymers were on the lower range, as smaller polymer chains degrade faster and hydration is accelerated due to a higher number of carboxylic acid end groups[51–53]. This approach was supported by the observation of a nearly complete in vitro release of 87% for RG 502 H-Big which was achieved within three days, whereas only 69% drug was released for RG 503 H-Big after one week. It is interesting to note that larger particles showed a higher burst release despite their smaller specific surface area. It is tempting to speculate that buprenorphine is not evenly distributed throughout particles and that bigger particles might therefore have higher surface bound drug concentrations. However, exploratory results using energy-dispersive X-ray microanalysis failed to provide distinct distribution patterns of drug within particles due to melting of PLGA during irradiation. Other polymers regularly used for sustained-release formulations like PCL or PLA were not considered for this work due to their unfavorable and slow release characteristics.

A burst release can be attributed to drug solubilization from or close to the surface of the carrier. As this is often regarded as unfavorable in achieving a consistent and continuous release profile[54], a burst release of around 30% was favorable in the present study, to ensure full analgesic effect immediately after administration. With a burst and cumulative release over three days of 28% and 87%, respectively, formulation RG 502 H-Big was most suitable for all further studies. An alternative formulation for use in vivo could be RG 502 H-Small with a burst release of 16% and complete in vitro release between 48 and 72 h. In such a scenario, due to the lower burst release, an additional loading dose would be needed. However, such an approach would complicate handling and formulation preparation. Therefore, formulation RG 502 H-Big was used for all in vivo studies.

The amount of BUP for the depot formulation was chosen based on the standard therapeutic dose for mice, which is 0.1 mg/kg every 6 h. Hence, 12 injections are necessary resulting in a total dose of 1.2 mg/kg for three days. Accordingly, a total dose of 1.2 mg/kg BUP of the sustained-release formulation was administered to cover the same period. Pharmacokinetics of the depot formulation were assessed through plasma-and brain-concentration–time profiles and compared to non-retard BUP. Previous studies have shown therapeutic effective concentrations of BUP in plasma with a threshold of around 1 ng/mL for rodents[55,56]. In the present study, a single injection of non-retarded BUP resulted in plasma concentrations above 1 ng/mL only at the first measured time point at 2 h. At 12 h plasma levels declined considerable (0.4 ng/mL) and are almost not detectable at 24 h, since 5 out of 7 mice showed values below the LLOQ. The presented findings correlate with previous studies, indicating that a non-retard dose of 0.1 mg/kg results in high enough BUP plasma concentrations for a couple of hours only[43,57,58]. The described burst release of the depot formulation contributed to the fast rise and decline of drug levels in the first hours, which represented the first phase of the pharmacokinetic profile. Thereafter, the sustained-release formulation showed a second phase, including plasma levels close to 1 ng/mL for the duration of 12 h and a detectable concentration of drug until the last measurement point at 72 h, demonstrated that a sustained drug release was achieved.

While the threshold of 1 ng/mL is often referenced for assessing analgesic action of buprenorphine, this only takes plasma or blood levels into account[55,56]. As an opioid derivative, buprenorphine executes its analgesic effect through various opioid (μ-/κ-/δ-) receptors in the brain[59,60]. Ohtani et al. have shown that a good correlation exists between specific binding concentrations of BUP in the brain and analgesic effects in rats. Furthermore, analgesic activity and plasma concentrations did not correlate, especially in the early phase after administration[61]. Therefore, brain exposure of BUP over time was analyzed and correlated to the analgesic effect shown in the

thermal sensitivity assay. Contrary to the plasma profiles, which dropped below 1 ng/ml, a single injection of the sustained-release formulation lead to an elevated specific binding concentration in the brain of 2.3 ng/g until the last measurement point at 72 h. While after injection of the non-retarded formulation the specific binding concentration of BUP in the brain showed a higher exposure compared to plasma level after 12 h, the concentration in the brain decreased significantly thereafter. Taking the results from the thermal sensitivity assay into account, specific binding concentrations of BUP in the brain of 3 ng/g might not be enough to alleviate pain reliably, as can be seen for mice receiving non-retard solution after 12 h (Fig. 4a). Withdrawal latencies at that time point show still higher values with 14.3 s for non-retard formulation compared to 11.3 s for control animals but no significant difference can be found. In contrast to that, animals receiving depot formulation showed significantly increased withdrawal latencies even 24 h post injection, which correlates to drug concentrations of around 5 ng/g in brain. It could be speculated that specific binding concentration values of 5 ng/g in the brain need to be achieved to reliably relieve pain in mice. Therefore, it could be expected that one injection of depot formulation alleviates strong pain only up to 24 h. Withdrawal latencies measured 48 h post injection showed still high values but no significant differences could be established to control animals at the 24 h time point , which correlates to specific binding concentrations of less than 3 ng/g at 48 and 72 h post injection. Analgesic performance determined through thermal sensitivity assay confirmed the short duration of action of the non-retarded formulation shown previously[11,16,43]. In line with plasma and brain concentration profiles, one injection of the non-retarded solution resulted only at the first 2 h time point in significantly higher withdrawal latencies (Fig. 4).

Buprenorphine is a potent analgesic drug and is therefore used to alleviate moderate to severe pain caused by e.g. surgeries[48]. Consequently, effectiveness and adverse effects have to be judged after clinically relevant, i.e. surgical, pain stimuli. In the present study, a sham ovariectomy was performed, which caused mild to moderate pain for less than 24 h[48,62]. One subcutaneous injection of sustained-release formulation was compared to two injections of a non-retarded BUP solution. Results confirmed the findings of the hot plate assay.

Since the maximal plasma and brain levels for both formulations were comparable, no difference in systemic side effects like body weight reduction, decreased food and water intake, and nesting behavior[44,48,63–65] were anticipated. In particular, decrease in food and water intake, and thereof reduction of body weight, are widely described after surgeries but are also well-known side effects of opioids[16,63,66,67]. Both formulations led to a decreased food intake and body weight 24 h after surgery but did not differ from each other. Other studies have shown, that increased handling stress due to recurring injections can lead to a further decrease in body weight or food intake[43]. Since animals treated with the non-retarded solution received only two injections, handling stress was only minimal and therefore not expected to result in differences between the protocols. No further adverse events have been noted regarding sustained-release formulation, except two animals with local effects at injection site as described above. Nevertheless, further studies have to be conducted to confirm the safety profile of the depot formulation.

Buprenorphine is a well-established drug and has been on the market for decades, demonstrating safety and efficacy[68]. However, pain is not only a highly inter-individual variable, but recent research demonstrates that pain mediation differs depending on mouse strain and sex[69,70]. For the present study, only female C57BL/6 J mice were used. It remains to be elucidated if buprenorphine doses have to be adjusted for other mouse strains, rats, or males.

In conclusion, a novel depot formulation of buprenorphine based on biodegradable PLGA microparticles was successfully developed. Specific binding concentrations of BUP in the brain as the target site of opioids show therapeutic levels of drug for at least 24 h and a trend towards pain relief for at least 72 h. Thermal sensitivity assay confirms analgesic effect for at least 24 h, which is a significantly prolonged duration of action compared to the marketed standard formulation. In a proof-of-concept, post-surgical pain relief was accomplished in sham ovariectomy without notable side effects. The presented buprenorphine depot formulation offers a more efficient and less stressful pain relief for laboratory mice and can therefore be considered a refinement of current analgesic protocols. A possible use of the new buprenorphine depot formulation for companion animals, such as cats and dogs, would considerably widen the field of application.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Received: 20 May 2020; Accepted: 28 September 2020
Published online: 14 October 2020

References

1. Buprenorphine: a reappraisal of its antinociceptive effects and therapeutic use in alleviating post-operative pain in animals. *Lab. Anim.***36**, 322–343 (2002).
2. Cowan, A. Buprenorphine: the basic pharmacology revisited. *J. Addict. Med.* **1**, 68 (2007).
3. Cowan, A., Lewis, J. W. & Macfarlane, I. R. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. *Br. J. Pharmacol.* **60**, 537–545 (1977).
4. Dahan, A. *et al.* Comparison of the respiratory effects of intravenous buprenorphine and fentanyl in humans and rats. *BJA Br. J. Anaesth.* **94**, 825–834 (2005).
5. Dahan, A. *et al.* Buprenorphine induces ceiling in respiratory depression but not in analgesia. *BJA Br. J. Anaesth.* **96**, 627–632 (2006).
6. Ohtani, M., Kotaki, H., Nishitateno, K., Sawada, Y. & Iga, T. Kinetics of respiratory depression in rats induced by buprenorphine and its metabolite Norbuprenorphine. *J. Pharmacol. Exp. Ther.* **281**, 428–433 (1997).
7. Stokes, E. L., Flecknell, P. A. & Richardson, C. A. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. *Lab. Anim.* **43**, 149–154 (2009).

8. Gopal, S., Tzeng, T.-B. & Cowan, A. Characterization of the pharmacokinetics of buprenorphine and norbuprenorphine in rats after intravenous bolus administration of buprenorphine. *Eur. J. Pharm. Sci.* **15**, 287–293 (2002).
9. Ohtani, M., Kotaki, H., Uchino, K., Sawada, Y. & Iga, T. Pharmacokinetic analysis of enterohepatic circulation of buprenorphine and its active metabolite, norbuprenorphine, in rats. *Drug Metab. Dispos. Biol. Fate Chem.* **22**, 2–7 (1994).
10. Yu, S. *et al.* Pharmacokinetics of Buprenorphine after Intravenous Administration in the Mouse. *J. Am. Assoc. Lab. Anim. Sci.* **45**, 12–16 (2006).
11. Gades, N. M., Danneman, P. J., Wixson, S. K. & Tolley, E. A. The Magnitude and duration of the analgesic effect of morphine, butorphanol, and buprenorphine in rats and mice. https://www.ingentaconnect.com/content/aalas/jaalas/2000/00000039/00000002/art00001# (2000).
12. Effect of restraint and injection methods on heart rate and body temperature in mice. *Lab. Anim.***40**, 382–391 (2006).
13. Girón, R. *et al.* Synthesis and opioid activity of new fentanyl analogs. *Life Sci.* **71**, 1023–1034 (2002).
14. Jagerovic, N. *et al.* Long-acting fentanyl analogues: synthesis and pharmacology of N-(1-Phenylpyrazolyl)-N-(1-phenylalkyl-4-piperidyl)propanamides. *Bioorg. Med. Chem.* **10**, 817–827 (2002).
15. Evangelista-Vaz, R., Bergadano, A., Arras, M., Jirkof, P. D. Analgesic efficacy of subcutaneous–oral dosage of tramadol after surgery in C57BL/6J mice. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***57**, 368–375 (2018).
16. Sauer, M., Fleischmann, T., Lipiski, M., Arras, M. & Jirkof, P. Buprenorphine via drinking water and combined oral-injection protocols for pain relief in mice. *Appl. Anim. Behav. Sci.* **185**, 103–112 (2016).
17. Peterson, N. C., Nunamaker, E. A. & Turner, P. V. To Treat or Not to Treat: The Effects of Pain on Experimental Parameters. https://www.ingentaconnect.com/content/aalas/cm/2017/00000067/00000006/art00002# (2017).
18. Bode, C., Kranz, H., Fivez, A., Siepmann, F. & Siepmann, J. Often neglected: PLGA/PLA swelling orchestrates drug release: HME implants. *J. Controlled Release* **306**, 97–107 (2019).
19. Bode, C., Kranz, H., Siepmann, F. & Siepmann, J. In-situ forming PLGA implants for intraocular dexamethasone delivery. *Int. J. Pharm.* **548**, 337–348 (2018).
20. Budhian, A., Siegel, S. J. & Winey, K. I. Production of haloperidol-loaded PLGA nanoparticles for extended controlled drug release of haloperidol. *J. Microencapsul.* **22**, 773–785 (2005).
21. Jain, R. A., Rhodes, C. T., Railkar, A. M., Malick, A. W. & Shah, N. H. Controlled release of drugs from injectable in situ formed biodegradable PLGA microspheres: effect of various formulation variables. *Eur. J. Pharm. Biopharm.* **50**, 257–262 (2000).
22. Wei, Y., Wang, Y., Zhang, H., Zhou, W. & Ma, G. A novel strategy for the preparation of porous microspheres and its application in peptide drug loading. *J. Colloid Interface Sci.* **478**, 46–53 (2016).
23. June 15, P. & Tweet, 2016. FDA's Regulatory Science Program for Generic PLA/ PLGA-Based Drug Products. https://www.americanpharmaceuticalreview.com/Featured-Articles/188841-FDA-s-Regulatory-Science-Program-for-Generic-PLA-PLGA-Based-Drug-Products/.
24. Rosenthal, R. N. & Goradia, V. V. Advances in the delivery of buprenorphine for opioid dependence. *Drug Des. Devel. Ther.* **11**, 2493–2505 (2017).
25. Carbone, E. T., Lindstrom, K. E., Diep, S. & Carbone, L. Duration of action of sustained-release buprenorphine in 2 strains of mice. *J. Am. Assoc. Lab. Anim. Sci.* **51**, 815–819 (2012).
26. Healy, J. R. *et al.* Evaluation of an improved sustained-release buprenorphine formulation for use in mice. *Am. J. Vet. Res.* **75**, 619–625 (2014).
27. Traul, K. A. *et al.* Safety studies of post-surgical buprenorphine therapy for mice. *Lab. Anim.* **49**, 100–110 (2015).
28. Chum, H. H. *et al.* Antinociceptive effects of sustained-release buprenorphine in a model of incisional pain in rats (Rattus norvegicus). *J. Am. Assoc. Lab. Anim. Sci. JAALAS***53**, 193–197 (2014).
29. Foley, P. L., Liang, H. & Crichlow, A. R. Evaluation of a sustained-release formulation of buprenorphine for analgesia in rats. *J. Am. Assoc. Lab. Anim. Sci.* **50**, 198–204 (2011).
30. Johnson, R. A. Voluntary running-wheel activity, arterial blood gases, and thermal antinociception in rats after 3 buprenorphine formulations. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***55**, 306–311 (2016).
31. Cary, C. D. *et al.* Pharmacokinetic profiles of meloxicam and sustained-release buprenorphine in prairie dogs (Cynomys ludovicianus). *J. Am. Assoc. Lab. Anim. Sci.* **56**, 160–165 (2017).
32. Catbagan, D. L., Quimby, J. M., Mama, K. R., Rychel, J. K. & Mich, P. M. Comparison of the efficacy and adverse effects of sustained-release buprenorphine hydrochloride following subcutaneous administration and buprenorphine hydrochloride following oral transmucosal administration in cats undergoing ovariohysterectomy. *Am. J. Vet. Res.* **72**, 461–466 (2011).
33. Molter, C. M. *et al.* Pharmacokinetics of a single subcutaneous dose of sustained release buprenorphine in northern elephant seals (Mirounga angustirostris). *J. Zoo Wildl. Med. Off. Publ. Am. Assoc. Zoo Vet.***46**, 52–61 (2015).
34. Nunamaker, E. A., Goldman, J. L., Adams, C. R. & Fortman, J. D. Evaluation of Analgesic Efficacy of Meloxicam and 2 Formulations of Buprenorphine after Laparotomy in Female Sprague–Dawley Rats. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***57**, 498–507 (2018).
35. Nunamaker, E. A. *et al.* Pharmacokinetics of 2 Formulations of Buprenorphine in Macaques (Macaca mulatta and Macaca fascicularis). *J. Am. Assoc. Lab. Anim. Sci.* **52**, 48–56 (2013).
36. Smith, B. J., Wegenast, D. J., Hansen, R. J., Hess, A. M. & Kendall, L. V. Pharmacokinetics and Paw Withdrawal Pressure in Female Guinea Pigs (Cavia porcellus) Treated with Sustained-Release Buprenorphine and Buprenorphine Hydrochloride. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***55**, 789–793 (2016).
37. Seymour, T. L. *et al.*Postoperative Analgesia Due to Sustained-Release Buprenorphine, Sustained-Release Meloxicam, and Carprofen Gel in a Model of Incisional Pain in Rats (Rattus norvegicus). *J. Am. Assoc. Lab. Anim. Sci. JAALAS***55**, 300–305 (2016).
38. Zullian, C. *et al.* Plasma concentrations of buprenorphine following a single subcutaneous administration of a sustained release formulation of buprenorphine in sheep. *Can. J. Vet. Res. Rev. Can. Rech. Veterinaire***80**, 250–253 (2016).
39. Clark, T. S., Clark, D. D. & Jr, R. F. H. Pharmacokinetic Comparison of Sustained-Release and Standard Buprenorphine in Mice. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***53**, 387–391 (2014).
40. Farzan, M. *et al.* Loading of Porous Functionalized Calcium Carbonate Microparticles: Distribution Analysis with Focused Ion Beam Electron Microscopy and Mercury Porosimetry. *Pharmaceutics***11**, (2019).
41. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals*. (National Academies Press (US), 2011).
42. Mähler (Convenor), M. *et al.*FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab. Anim.***48**, 178–192 (2014).
43. Jirkof, P., Tourvieille, A., Cinelli, P. & Arras, M. Buprenorphine for pain relief in mice: repeated injections vs sustained-release depot formulation. *Lab. Anim.* **49**, 177–187 (2015).
44. Jirkof, P. *et al.* Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring. *Lab. Anim.* **47**, 153–161 (2013).
45. Langford, D. J. *et al.* Coding of facial expressions of pain in the laboratory mouse. *Nat. Methods* **7**, 447–449 (2010).
46. Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175–191 (2007).
47. Pert, C. B. & Snyder, S. H. Opiate Receptor: Demonstration in Nervous Tissue. *Science* **179**, 1011–1014 (1973).
48. Arras, M., Rettich, A., Cinelli, P., Kasermann, H. P. & Burki, K. Assessment of post-laparotomy pain in laboratory mice by telemetric recording of heart rate and heart rate variability. *BMC Vet Res* **3**, 16 (2007).

49. Janoria, K. G. & Mitra, A. K. Effect of lactide/glycolide ratio on the in vitro release of ganciclovir and its lipophilic prodrug (GCV-monobutyrate) from PLGA microspheres. *Int. J. Pharm.* **338**, 133–141 (2007).
50. Langer, R. & Chasin, M. Biodegradable polymers as drug delivery systems. *Inf. Health Care* (1990).
51. Eliaz, R. E. & Kost, J. Characterization of a polymeric PLGA-injectable implant delivery system for the controlled release of proteins. *J. Biomed. Mater. Res.* **50**, 388–396 (2000).
52. Mittal, G., Sahana, D. K., Bhardwaj, V. & Ravi Kumar, M. N. V. Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. *J. Controlled Release***119**, 77–85 (2007).
53. Ravivarapu, H. B., Burton, K. & DeLuca, P. P. Polymer and microsphere blending to alter the release of a peptide from PLGA microspheres. *Eur. J. Pharm. Biopharm.* **50**, 263–270 (2000).
54. Huang, X. & Brazel, C. S. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *J. Controlled Release* **73**, 121–136 (2001).
55. Yassen, A., Olofsen, E., Dahan, A. & Danhof, M. Pharmacokinetic-pharmacodynamic modeling of the antinociceptive effect of buprenorphine and fentanyl in rats: role of receptor equilibration kinetics. *J. Pharmacol. Exp. Ther.* **313**, 1136–1149 (2005).
56. Yun, M.-H., Jeong, S.-W., Pai, C.-M. & Kim, S.-O. Pharmacokinetic-Pharmacodynamic modeling of the analgesic effect of buprederm™, in mice. *Health (N. Y.)***02**, 824 (2010).
57. Clark, T. S., Clark, D. D. & Hoyt, R. F. Pharmacokinetic Comparison of Sustained-Release and Standard Buprenorphine in Mice. https://www.ingentaconnect.com/content/aalas/jaalas/2014/00000053/00000004/art00008# (2014).
58. Kendall, L. V. *et al.*Pharmacokinetics of Sustained-Release Analgesics in Mice. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***53**, 478–484 (2014).
59. Sadée, W., Rosenbaum, J. S. & Herz, A. Buprenorphine: differential interaction with opiate receptor subtypes in vivo. *J. Pharmacol. Exp. Ther.* **223**, 157–162 (1982).
60. Villiger, J. W. & Taylor, K. M. Buprenorphine: Characteristics of binding sites in the rat central nervous system. *Life Sci.* **29**, 2699–2708 (1981).
61. Ohtani, M., Kotaki, H., Sawada, Y. & Iga, T. Comparative analysis of buprenorphine- and norbuprenorphine-induced analgesic effects based on pharmacokinetic-pharmacodynamic modeling. *J. Pharmacol. Exp. Ther.* **272**, 505–510 (1995).
62. Roughan, J. V. & Flecknell, P. A. Behaviour-based assessment of the duration of laparotomy-induced abdominal pain and the analgesic effects of carprofen and buprenorphine in rats. *Behav. Pharmacol.* **15**, 461–472 (2004).
63. Hayes, K. E., Raucci, J., Gades, N. M. & Toth, L. A. An evaluation of analgesic regimens for abdominal surgery in mice. https://www.ingentaconnect.com/content/aalas/jaalas/2000/00000039/00000006/art00004 (2000).
64. Jirkof, P. Burrowing and nest building behavior as indicators of well-being in mice. *J Neurosci Methods* **234**, 139–146 (2014).
65. Recognizing and assessing pain. suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations. *Lab. Anim.* **36**, 378–395 (2002).
66. Adamson, T. W. *et al.* Assessment of Carprofen and buprenorphine on recovery of mice after surgical removal of the mammary fat pad. *J. Am. Assoc. Lab. Anim. Sci. JAALAS***49**, 610–616 (2010).
67. Desborough, J. P. The stress response to trauma and surgery. *Br. J. Anaesth.* **85**, 109–117 (2000).
68. Christoph, T. *et al.* Broad analgesic profile of buprenorphine in rodent models of acute and chronic pain. *Eur. J. Pharmacol.* **507**, 87–98 (2005).
69. Sorge, R. E. *et al.* Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male But Not Female Mice. *J. Neurosci.* **31**, 15450–15454 (2011).
70. Sorge, R. E. *et al.* Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat. Neurosci.* **18**, 1081–1083 (2015).

Acknowledgements

The authors would like to thank Dr. Peter Wipfli and Claudia Textor from Novartis for providing and supporting LC-MS analysis of plasma and brain samples. The authors thank Evi Bieler and Daniel Mathys from Nano Imaging Lab, SNI, University of Basel for their support for scanning electron microscopy. The authors thank furthermore Dr. Arel Su (Roche), Dr. Elvire Fournier (Idorsia), Dr. Michaela Thallmair (UZH) and the BZL staff (USZ) for their support as well as the many colleagues from industry and regulatory authorities for their feed-back and support.

Author contributions

V.S. carried out all experiments and prepared all figures and tables. M.D. helped with all animal experiments. Dr. M.A. was involved in planning of animal studies. Dr. P.D. helped with pharmacokinetic studies and was involved in planning and supervision of the project. Dr. P.J. and Prof. Dr. J. H. have equally contributed to the present work and jointly devised and supervised the project. All authors have reviewed the manuscript and agree with its contents.

Funding

Financial support for the project was obtained by grants from Unitectra (University of Basel), University of Zürich, Interpharma Basel, F. Hoffmann-La Roche Ltd Basel, and Novartis Ltd Basel.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-74230-6.

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Supplement

Table S1: ***Number of mice used for different experimental set ups.***

1. Mice used for hotplate assay and for PK study (Mice were only used once)	
Group	**Number of Mice**
BUP-Depot	24
Non-retard Formulation	18
NaCl	0
2. Mice used for PK only	
Group	**Number of Mice**
BUP-Depot	21
Non-retard Formulation	2
NaCl	2
3. Mice used only for hotplate assay once	
Group	**Number of Mice**
BUP-Depot	15
Non-retard Formulation	0
NaCl	1
4. Mice used for hotplate assay twice [a]	
Group	**Number of Mice**
First Round	
Non-retard Formulation	22
NaCl	9
Second Round	
BUP-Depot	9
Non-retard Formulation	9[b]
NaCl	13[b]
5. Surgical procedure [b]	
Group	**Number of Mice**
BUP-Depot	6
Non-retard Formulation	6

[a] *A total of 31 mice were used twice for the hotplate assay. After first round, mice were allocated randomly to new groups after a wash-out phase of 6 days to a second round.*

[b] Mice that were used twice for the hotplate assay and received only non-retard formulation or NaCl were used after a wash-out phase of 41 days for surgical procedures.

Table S2: ***Number of mice per group with average weight and average applied dose used for pharmacokinetic study.*** *Data is presented as ± SD.*

Group	Number of Mice	Time Points	Number of Mice per Time Point	Weight [g]	Dose [mg/kg]
BUP-Depot	45	0.5, 2, 5, 72 hours 12, 24, 48 hours	6 7	17.7 ± 1.4	1.1 ± 0.04
Non-retard Formulation	22	2 hours 12, 24 hours	6 7	17.2 ± 1.9	0.12 ± 0.01
NaCl	2	-	-	19.0	-

Table S3: ***Number of mice per group and time point with average weight and average applied dose used for hotplate assay.*** *Data presented as ± SD.*

Group	Number of Mice per Time Point	Time Points	Weight [g]	Dose [mg/kg]
BUP-Depot	12 11	2, 12, 48 hours 24 hours	18.3 ± 1.3	1.1 ± 0.1
Non-retard Formulation	12	2, 12, 24 hours	18.1 ± 1.8	0.11 ± 0.01
NaCl	12	2, 12, 24 hours	18.8 ± 1.8	-

Table S4: ***Number of mice per group with average weight and average administered dose used for surgical procedure (sham ovariectomy).*** *Data presented as ± SD.*

Group	Number of Mice	Weight [g]	Dose [mg/kg]
BUP-Depot	6	21.8 ± 1.3	1.1 ± 0.1
Non-retard Formulation	6	22.2 ± 1.2	0.11 ± 0.002

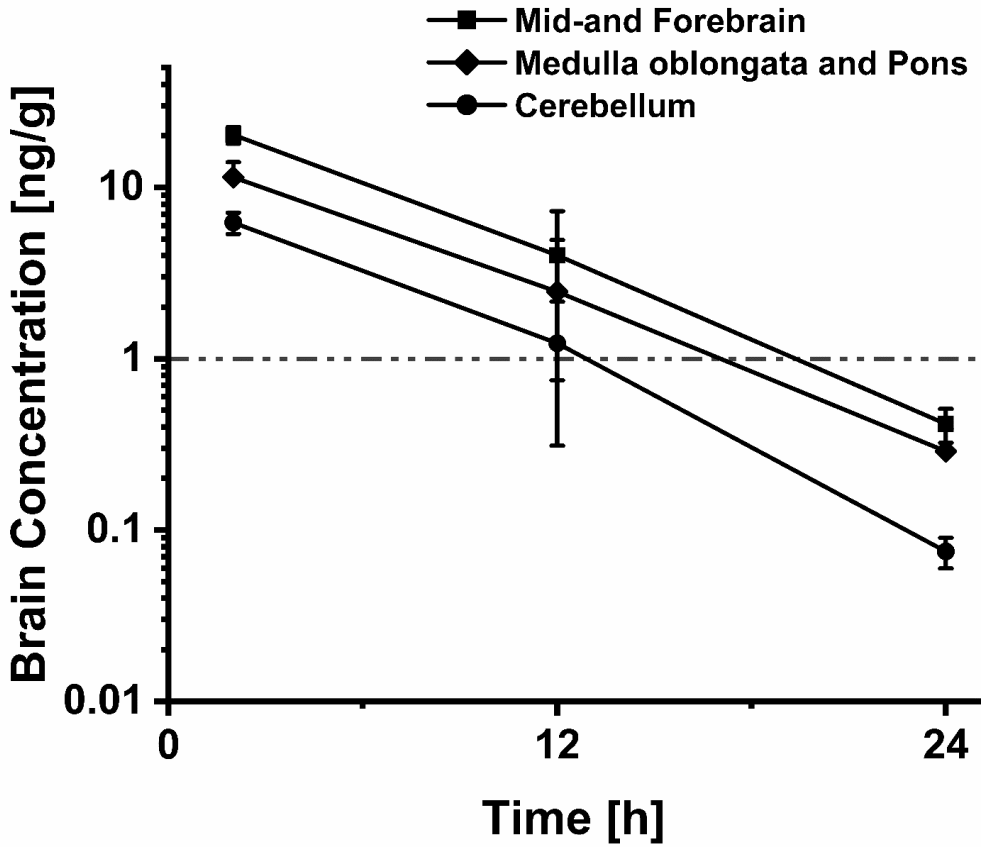

Figure S1: ***Regional brain concentration-time profile of non-retard formulation after single subcutaneous injection (0.1 mg/kg)****. Dashed line represents 1 ng/g and data is expressed as mean ± SD.*

Buprenorphine Loaded PLGA Microparticles:

Characterization of a Sustained-Release Formulation

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Personal contribution:
My contribution to this research article includes the design, planning and conducting of all experiments. Furthermore, I evaluated the results and prepared all figures and tables. I wrote the manuscript with the help of Jörg Huwyler, Paulin Jirkof and Pascal Detampel.

Journal of Drug Delivery Science and Technology 63 (2021) 102558

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Buprenorphine loaded PLGA microparticles: Characterization of a sustained-release formulation

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ARTICLE INFO

Keywords:
Microparticles
PLGA
Buprenorphine
X-ray sterilization
Depot formulation

ABSTRACT

Buprenorphine is a short-acting analgesic drug. Its use in veterinary medicine requires several injections per day to alleviate pain in animals. We, therefore, designed a poly-lactic-*co*-glycolic acid (PLGA) based depot formulation of the opioid to prolong the analgesic effect. We characterized our novel microparticulate depot formulation with emphasis on a potential future product development in the present work.

Microparticles showed low residual moisture levels and did meet bacterial endotoxin requirements defined by the European Pharmacopeia. Depot formulation showed physicochemical stability over 6 months at 4 °C, indicating sufficient shelf life. Reconstituted formulation stored at 4 °C showed unchanged release properties for 24 h. Terminal sterilization by x-rays with a dose of 30 kGy revealed the sensitivity of buprenorphine towards radiation, resulting in substantial drug degradation. However, microparticle properties were not affected. Our experiments indicate that sterilization of the final product is not possible, therefore requiring aseptic manufacturing protocols.

In conclusion, our novel buprenorphine depot formulation based on PLGA microparticles can be stored for at least 6 month as a lyophilisate and can be used for at least 24 h after reconstitution. Pilot experiments suggest that scale-up and a future aseptic production should be feasible. We conclude that the novel depot formulation shows promising attributes, a prerequisite for future industrial production and commercialization.

1. Introduction

Controlled release formulations is a modern tool to achieve a prolonged and consistent drug release. Thus, application intervals, plasma exposure peaks, as well as side effects are reduced. Research has been going on for many decades and significant advances have been made since the first oral controlled release formulation in 1952 [1]. Since then, the development of biodegradable polymers like polylactic acid, poly (glycolic acid), and poly-lactic-*co*-glycolic acid (PLGA), which were first introduced as suture materials [2,3], resulted in considerable advancements in controlled parenteral drug delivery. PLGA is one of the most studied synthetic polymers used for sustained release in the last decades. Its use for controlled drug delivery has been studied extensively, culminating in the approval as an excipient by the US Food and Drug Administration and the European Medicines Agency [4,5]. Marketed controlled release formulations using PLGA as excipient consist primarily of subcutaneous applied microparticles or subcutaneous *in-situ* forming implants [6–9]. It's biodegradable and biocompatible characteristics [10–12] as well as its long history of clinical use make it very attractive for formulation development. The duration of action can thereby be prolonged from weeks up to months [13–20]. Application areas are equally versatile, including bone regeneration, cancer treatment, pain and addiction management, and vaccination [20–24]. The first PLGA based drug products were already approved in the 1980s by the US Food and Drug Administration. Considerable research was done since then to establish PLGA based formulations. However, only about 19 PLGA containing drug products are currently on the market [4,5,25]. Several problems were identified in connection with PLGA based delivery platforms regarding production and storage. For one, drug products based on biodegradable polymers need to be stored in a water-free environment. High moisture levels can lead to premature hydrolysis of the polymeric backbone, altering drug release and reducing stability of the active compound [26–28]. Therefore, an optimized drying procedure is essential to ensure low residual moisture levels, which enables

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https://doi.org/10.1016/j.jddst.2021.102558
Received 2 March 2021; Received in revised form 28 April 2021; Accepted 28 April 2021
Available online 4 May 2021

sufficient shelf life. Another major issue is the required sterility of parenteral drugs [29]. Dry or moist heat would damage the product and other procedures like sterile filtration are not possible. Therefore, sterilization strategies for PLGA formulations are mostly limited to terminal sterilization through irradiation. Another option is aseptic processing. However, previous studies showed that polymers like PLGA undergo chain scission and molecular weight reduction if subjected to gamma or electron beam irradiation [30–34]. Thereby, properties like drug release and morphology of drug carriers may change [30,33]. Working under aseptic environment is therefore in most cases necessary and the only option.

Buprenorphine is a small molecule with limited oral bioavailability, making it an optimal candidate for the parenteral route [35,36]. As an immediate acting injection solution, it is frequently used in veterinary medicine for pain management [37,38]. However, due to its short terminal half-life of around 3 h in mice and rats [39–41], several injections per day are necessary [41,42]. Repeated administrations in animals are challenging, as this route of application causes additional pain and increases handling associated stress [43]. Other depot formulations for veterinary use were previously developed, e.g. drug suspension based on cholesterol, glycerol tristearate and medium-chain triglyceride oil [44,45] (Animalgesics for Mice; Animalgesic Laboratories Inc., Millersville, MD) or a polymeric *in situ* forming implant [46] (Buprenorphine SR-Lab; ZooPharm, Fort Collins, CO). Both formulations showed a prolonged duration of action in rodents [45–47]. However, these formulations are not available in Europe and show several shortcomings, as discussed previously [48], such as inflammation and necrosis at the injection site, small injection volumes, high dose, and high viscosity of the solution. Therefore, we have designed a novel, size-controlled, safe, and effective microparticulate buprenorphine depot formulation based on PLGA [48].

The aim of the current study was to characterize our novel PLGA based buprenorphine formulation with a focus on future product development. This aim includes the study of shelf life, residual moisture, endotoxin content, and the impact of irradiation for terminal sterilization. Analysis of drug release during storage of reconstituted particles did allow us to define the optimal time frame of usage. This information is needed to prepare the ground for a market introduction and widespread use in veterinary medicine.

2. Materials and methods

2.1. Materials

Buprenorphine HCl (BUP) was obtained from Macfarlan Smith Limited (Edinburgh, United Kingdom) in EP/USP grade quality. Polyvinylalcohol (PVA; $M_w \approx 67{,}000$; 86.7–88.7 mol% hydrolysis), sucrose and tris buffer were purchased from Sigma Aldrich (St. Louis, MO). PLGA (Resomer RG 502 (inherent viscosity (IV) = 0.20–0.22 dL/g), Resomer RG 502H (IV = 0.20–0.22 dL/g), Resomer RG 503H (IV = 0.38 dL/g) was obtained from Evonik (Essen, Germany). N,N-Dimethylformamide, methanol, dichloromethane (DCM), ammonium acetate, sodium lauryl sulfate (SDS), and Karl-Fischer reagent were obtained from Roth (Karlsruhe, Germany). HEPES was from PanReac Apllichem (Darmstadt, Germany), and sodium chloride (NaCl) and acetic acid (glacial) were from Merck (Darmstadt, Germany). Limulus amebocyte lysate (LAL, Pierce Chromogenic Endotoxin Quant Kit) was purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Microparticle preparation

Microparticles loaded with buprenorphine HCl were prepared as described previously [44]. The 20 mg batch size of BUP for the depot formulation was chosen to allow for a therapeutic dose for mice of 1.2 mg/kg and a subcutaneous injection volume 5 μL/g. The 100 mg amount of polymer (PLGA) in combination with sucrose was adjusted to allow for slow drug release and to provide sufficient volume to the lyophilization cake. If the BUP-Depot formulation is to be used for other animal species, an adjustment of the BUP to polymer and sucrose ratio is suggested. For the preparation, PLGA (RG 502H, 100 mg) and BUP (5 mg) were dissolved in 5 mL of DCM. DCM was slowly added at a flow rate of 0.7 mL/min to 75 mL of ice-cooled 1% (w/v) PVA aqueous solution, consisting of 1 M NaCl and 25 mM tris buffer, pH 9. Emulsificaton was achieved by high speed stirring at 600 rpm (big particles) or 1200 rpm (small particles) with a three-bladed propeller stirrer (RW 16 basic, IKA-Werke, Staufen, Germany) for 15 min. Residual DCM was evaporated overnight under mild agitation at ambient temperature and atmospheric pressure. Microparticles were collected by centrifugation (21000 *g*, 10 min) and washed three times with Milli Q water. After the addition of sucrose (3.3 mg/mL), particles were lyophilized for 24 h using a pilot-scale freeze dryer (Epsilon 2-6D LSCplus; Christ, Osterode, Germany). Particles for x-ray sterilization were prepared by the same method using the three polymers RG 502H, RG 503H, and RG 502 with a stirring speed of 600 rpm. Particles based on RG 502H were additionally produced in two different sizes (our lead formulation BUP-Depot/RG 502 H-Big, and RG 502 H-Small).

To avoid losses of the API during decanting and washing stages, the filtering stage has been employed after the production of the particles in a Mowiol/TRISMA solution. The liquid mixture containing freshly produced drug microparticles with PVA, Trisma, and DCM is transferred into a rotary evaporator to remove the DCM. The separation process runs under 699 mbar pressure and 50 °C temperature of the water bath for 1 h. After this stage, the liquid is cooled to room temperature and filtered under vacuum through a Büchner funnel with a 450 nm membrane filter. After forming a cake on the filter surface, the microparticles washed off by 64 mL of 4% (w/w) sucrose solution and further transferred into lyophilization vials, each containing 4 mL liquid. While filling the vials, the particle suspension is constantly stirred to prevent sedimentation.

To further minimize the losses of the active substance and streamline the production process, the filtering step was substituted with evaporation as an alternative phase separation method. This method is performed identically to the production with the filtering method until the filtering stage. After DCM removal in the rotary evaporator, the particle suspension is kept in the round flask. The pressure is further reduced to 300 mbar, and nitrogen gas is injected into the water vapor atmosphere above the surface of the liquid. The latter is necessary to speed up the evaporation process through a convective movement of the water vapors. This process continues until the liquid level drops below 60 mL. After that, the liquid is cooled down to room temperature and topped up with 85% sucrose solution and WFI to maintain 4% sucrose solution in a total resulting liquid volume of 64 mL. The resulting liquid is transferred into 16 lyophilization vials, each containing 4 mL fluid. While filling the vials, the particle suspension is constantly stirred to prevent sedimentation.

2.3. Microparticle characterization

2.3.1. Size and morphology

Laser diffraction was used to measure the size distribution of microparticles in water (Mastersizer X, Malvern, Worcestershire, United Kingdom). A lens suitable to detect particle sizes ranging from 1.2 to 600 μm was used and particle size was expressed as volume mean diameter.

Scanning electron microscopy (SEM) was used to analyze the morphology of BUP loaded microparticles. After sputter-coating of lyophilized particles with 20 nm gold (EM ACE600, Leica, Wetzlar, Germany), surface and inner structure were analyzed through focused ion-beam assisted scanning electron microscopy (FIB-SEM; Helios NanoLab 650, FEI, OR) as demonstrated previously [49]. Briefly, top layers of polymer were ablated through a focused gallium beam and cross-sections of particles were obtained by cutting through spheres.

2.3.2. Analytical methods and drug loading

Loading of BUP was determined as previously described [44]. As an alternative method to identify the drug content in the produced particles, the technique published in the European Pharmacopeia (Monograph 10.0/2017) suits every need. Column: 50 mm, diameter 4.6 mm; stationary phase 3,5 μm, octadecyl silica gel. Mobile phase A consists of 10 vol of acetonitrile (ACN) and 90 vol of potassium dihydrogen buffer. The latter is prepared by dissolving 5.44 g of potassium dihydrogen in 900 mL of water, adjusted to pH 4.5 with 5% (v/v) solution of orthophosphoric acid, and diluted to 1000 mL. Mobile phase B is pure ACN. The gradient is set up as follows: 0–2 min phase A is 89% (v/v), 2–12 min phase A is 89 to 64% (v/v), 12–15 min phase A is 64-41% (v/v), 15–20 min phase A is 41 to 39% (v/v). The flow rate is 2 mL/min. The injection volume is 5 μl. Detection: UV spectrophotometer at 240 nm. Buprenorphine is eluting at 6.1 min. Ten known impurities are completely separated from the main peak and elute after 15 min.

Drug load (DL) in percentage was calculated as a ratio of measured drug content in sample to the total mass of a sample. Theoretical drug load in percentage was calculated as a ratio between total BUP against the total BUP and total PLGA used for this formulation. Encapsulation efficiency (EE) in percentage was defined as the ratio between DL and theoretical drug load.

2.3.3. Endotoxin content

Endotoxin content was determined using a LAL assay. Lyophilized particles were reconstituted with sterile 0.9% NaCl solution (B. Braun, Hessen, Germany) to obtain a similar concentration of BUP as used for previous mouse studies (~240 μg/mL). The supernatant was analyzed using a calibration curve ranging from 0.01 to 0.1 EU/mL. Limit for endotoxins in parenteral formulations was calculated by Equation [1]:

$$Limit\ [\frac{EU}{mL}] = \frac{K}{M}, \quad (1)$$

where K is defined as the threshold of pyrogenic dose per kg of body weight and is equal to 5 EU/kg if the administration is not intrathecal [50], and M is defined as maximum bolus dose per kg of body weight [mL/kg].

A calculated bolus dose of BUP for injection was based on previous mouse studies in our group. A single dose of 1.2 mg/kg was proposed and injection volume amounted to 5 mL/kg (M) body weight of mice [48].

2.3.4. Residual moisture

Residual moisture of lyophilized particles was analyzed using Karl-Fischer titration (703 KF Titrino, Metrohm AG, Herisau, Switzerland) with dry methanol using sodium tartrate dihydrate as a standard. A weighed amount of particles was reconstituted with 500 μl of dry methanol and incubated for a minimum of 10 min at room temperature. For water content determination, 100 μl of particle suspension was transferred to a reaction vessel and equilibrated for 2 min. The water content of dry methanol was likewise determined and subtracted from water content in a sample.

2.4. In vitro release study

In vitro release studies were carried out as described previously [48]. Briefly, lyophilized particles containing BUP were dispersed in 300 μL of 54 mM HEPES buffer, pH 7.4. 1.5% (w/v) SDS was added to the buffer since previous studies revealed that the addition of SDS at this concentration was necessary to allow for a complete dissolution of BUP. Each 300 μL sample represented one time-point. Samples were incubated at 37 °C and agitated at 250 revolutions per minute. At predefined time points, 260 μL of sample suspension was removed, centrifuged (21′000 *g*, 8 min), and the supernatant was chromatographically analyzed. BUP content in the remaining microparticle pellet was determined after dissolution in DMF. The cumulative release was normalized to the total amount of BUP in each sample. Burst release was defined as drug release within 1 h.

2.5. Shelf life of the lyophilisate

BUP loaded microparticles were characterized after lyophilization regarding drug load, particle size and burst release. The remaining particles were stored in crimped lyophilization vials at 4 °C and analyzed again after 1, 3, and 6 months. Storage at 2–8 °C is recommended since prolonged storage at room temperature leads to changes in microparticle morphology.

2.6. Shelf life of the reconstituted formulation

Lyophilized particles were reconstituted with 0.9% sterile saline solution to obtain a total BUP concentration between 170 and 230 μg/mL. After reconstitution, suspensions were vortexed and incubated without agitation at 4 °C for 1, 4 or 24 h. Subsequently, samples were distributed to Eppendorf tubes and centrifuged for 3 min at 21′000 *g*. The supernatant was filtered through a 0.45 μm PTFE membrane, and the released BUP was analyzed using the HPLC method. The remaining supernatant was discarded, and particles were resuspended in 54 mM HEPES buffer, pH 7.4. Release studies were carried out as described above.

Solubility of BUP in NaCl solution was determined by incubation of pure drug and a physical mixture of drug with polymer and sucrose together with sterile saline solution for 1 h at 4 °C. The simulated drug load of the physical mixture was between 4 and 5% (w/w). Solutions were filtered through 0.45 μm PTFE membranes after incubation and analyzed by a chromatographic method.

2.7. X-ray sterilization

Different PLGA types were used to prepare BUP loaded microparticles. All formulations were characterized before irradiation regarding drug load, particle size, burst release and cumulative drug release within 72 h. Particles were stored in crimped lyophilization vials at 4 °C. Sterilization was done by x-rays using a total dose of 30.4–30.8 kGy (Synergy Health, Däniken, Switzerland).

2.8. Statistical analysis

OriginPro 2018 software (OriginLab Ltd, MA) was used for statistical analysis. Data comparisons: ANOVA or paired two-tailed Student's t-test with a level of significance of $P < 0.05$. Values are means ± SD. Group sizes refer to series of measurements performed using a number n of different batches.

3. Results

3.1. Characterization of BUP loaded microparticles

Microparticle formulation RG 502 H-Big was used for all experiments. This formulation is referred to as "BUP-Depot" throughout this manuscript. For a justification of batch sizes and BUP to excipient ratios, see section 2.2. Properties of particles (n = 17 batches) were as follows: spheres had a size of 33.7 ± 10.9 μm, a drug load of 4.1 ± 1.2% (corresponding to an encapsulation efficiency of 86.1%) and a burst release of 29.5 ± 11.6%.

Formulation BUP-Depot was further assessed regarding residual moisture and endotoxin content in triplicate. Water content after lyophilization was 1.2 ± 0.5%, and endotoxin concentrations were 0.12 ± 0.03 EU/mL.

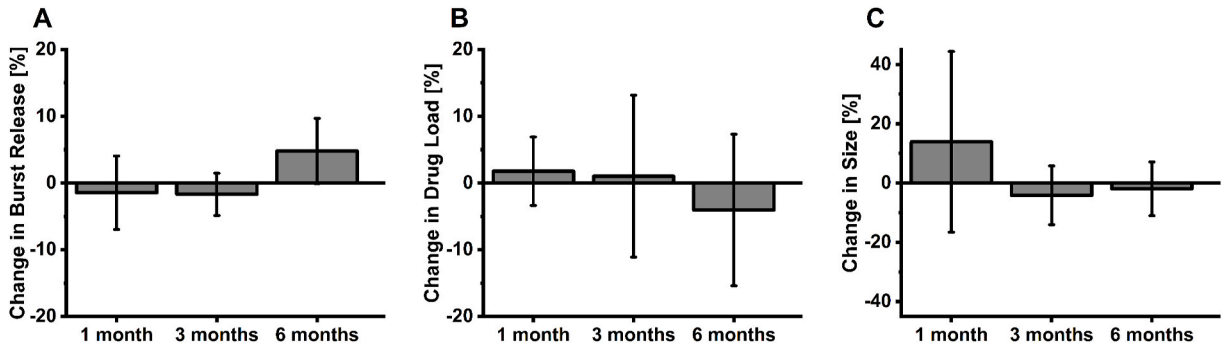

Fig. 1. Change in burst release, drug load and particle size after storage at 4°C for 1, 3 and 6 months. Statistical analysis revealed no significant changes over time. $P \geq 0.3$, $n = 3$.

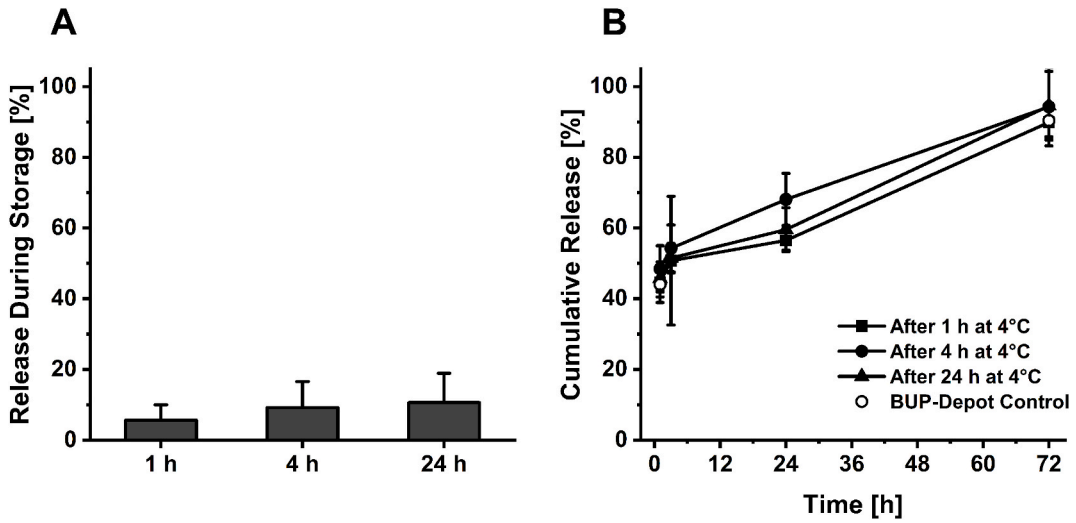

Fig. 2. Release of drug from reconstituted particles stored at 4°C. A) Reconstituted particles were kept at 4 °C in sterile 0.9% NaCl solution without agitation for the indicated period of time to determine total drug release during storage. B) Drug release over time at 37 °C in buffer pH 7.4 from particles previously stored at 4 °C. The same particles as shown in panel A were used. BUP-Depot Control: Particles without prior storage at 4 °C. No statistical differences between groups ($P \geq 0.5$). Data are means ± SD, $n \geq 3$.

3.2. Shelf life

Stability assessment of formulation BUP-Depot over a storage time of 6 months did include burst release, drug load, and size at day zero after lyophilization and after storage at 4 °C (Fig. 1). None of the parameters tested showed significant change ($P \geq 0.3$ for all samples) compared to control on day zero over a storage period of 6 months. In particular, burst release after storage (Fig. 1A) for 1, 3, and 6 months changed only by 1.8, 3.5, and 4.8%, respectively. Change in drug load (Fig. 1B) after 1, 3, and 6 months were minimal (1.8, 1.0 and 4.0%, respectively). Measurements of particle size showed no significant changes during 6 months (Fig. 1C).

3.3. Shelf life after reconstitution

Release of BUP from drug-loaded particles was analyzed at different time points after reconstitution with sterile 0.9% saline solution. Samples were incubated at 4 °C to simulate prolonged storage in a refrigerator (Fig. 2). Solubility in NaCl of pure drug and drug in a physical mixture with excipients (i.e. polymer and sucrose) was determined after 1 h at 4 °C to be 92.4 ± 1.3% and 93.7 ± 0.6%, respectively. The concentration of BUP in these and the following experiments was 218.7 ± 17.6 μg/mL. Incubations with BUP loaded particles for 1 h at 4 °C resulted in a drug release of 5.7%. More prolonged incubations for 4 h and 24 h resulted in a drug release of 9.2% and 10.6%, respectively (Fig. 2A). Almost complete drug release from particles was observed after 72 h only (i.e., 90.4%). BUP release from particles at 37 °C was similar for all particles, irrespective of the duration of preceding storage at 4 °C between 0 and 24 h (Fig. 2B).

3.4. X-ray sterilization

Drug release from microparticles was characterized with respect to the overall release kinetics (Fig. 3A) and the initial burst release (Fig. 3B). For qualitative assessment, release curves of different formulations were investigated for 168 h after irradiation [48]. It could be demonstrated that changes in polymer or particle size did hardly enhanced sensitivity towards x-rays. In particular, irradiation did not influence drug release. Cumulative release did depend on polymer type and particle size only and did not change after irradiation (Fig. 3C).

With respect to burst release (Fig. 3B), we did not observe significant changes except for formulation RG 502 H-Small, where burst decreased by 21% ($P = 0.031$).

In contrast to drug release, the drug load of particles decreased significantly after irradiation, as shown in Fig. 3D. Interestingly, big particles displayed higher drug losses (22%–27%) than smaller particles (12%). Assessment of particle size after irradiation revealed no significant changes as depicted in Fig. 3E. With respect to BUP-Depot and formulation RG 503H, formation of agglomerates (i.e. non-sintered particle assemblies) was observed after irradiation.

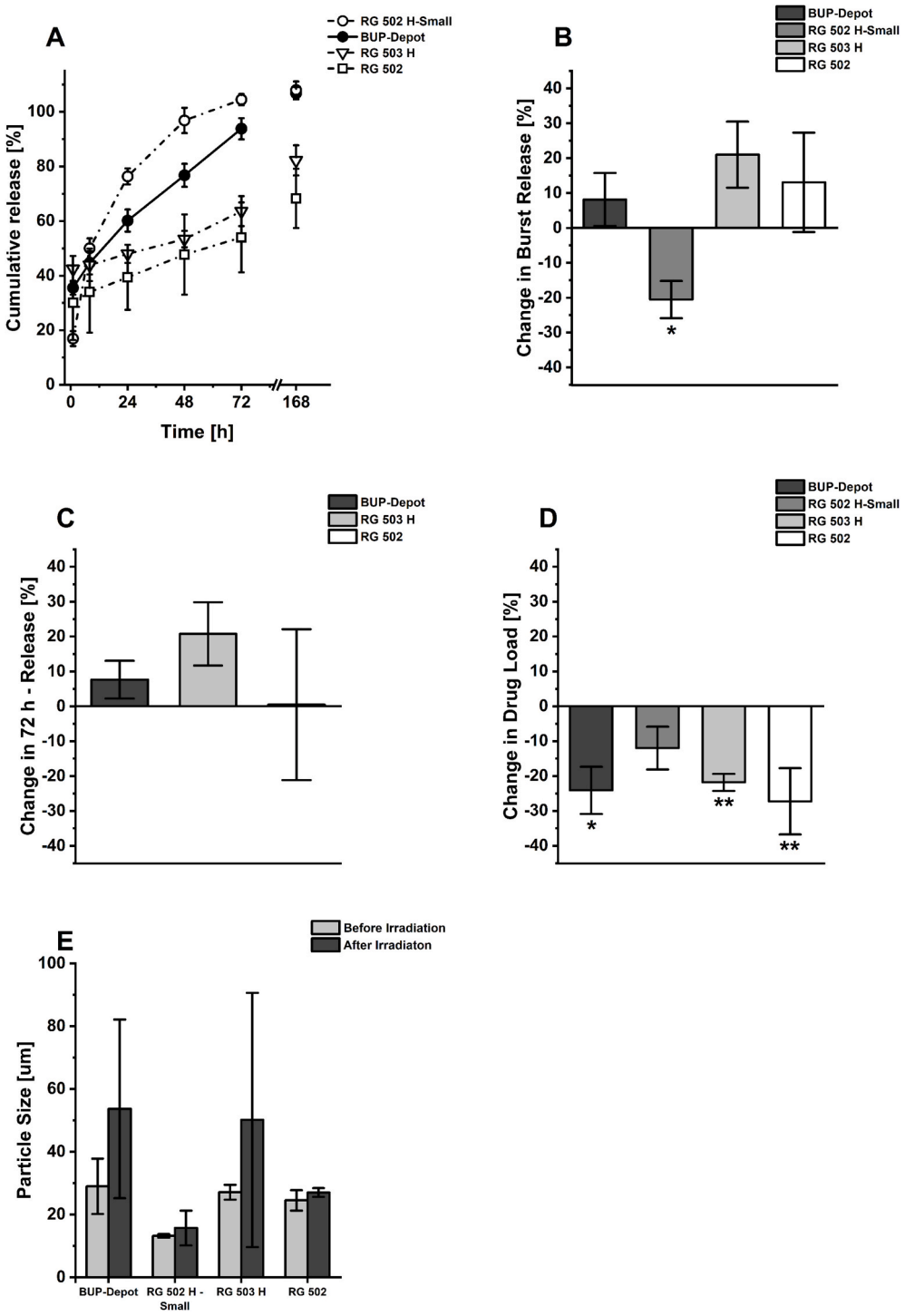

Fig. 3. Change of BUP loaded microparticle properties after x-ray. (A) In vitro release of BUP from different types of PLGA microspheres after irradiation. (B) Change in burst release. (C) Cumulative release within 72 h. RG 502 H-Small not shown since 100% release after 3 days. (D) Change in total drug load. (E) Particle size of different formulations before and after irradiation. Means ± SD, n = 3. (*: $P < 0.05$; **: $P < 0.01$).

Fig. 4 presents SEM pictures of BUP-Depot before and after x-ray treatment. Individual particle morphology did not change compared to untreated particles. Particles were still spherical with a homogenous and smooth surface (Fig. 4A and B). Examination of the particle core by FIB-SEM revealed no internal changes. Individual particles still had an intact and highly porous inner structure (Fig. 4C and D).

4. Discussion

Buprenorphine, an opioid derivative, is a veterinary drug that needs several administrations per day to alleviate pain reliably due to its short terminal half-life in rodents [39–41]. We have previously developed a novel size-controlled depot buprenorphine formulation for prolonged pain relief [48]. The goal of this study was to characterize our lead formulation, BUP-Depot, with the focus on suitability for future product development and manufacturing. The minor standard deviations,

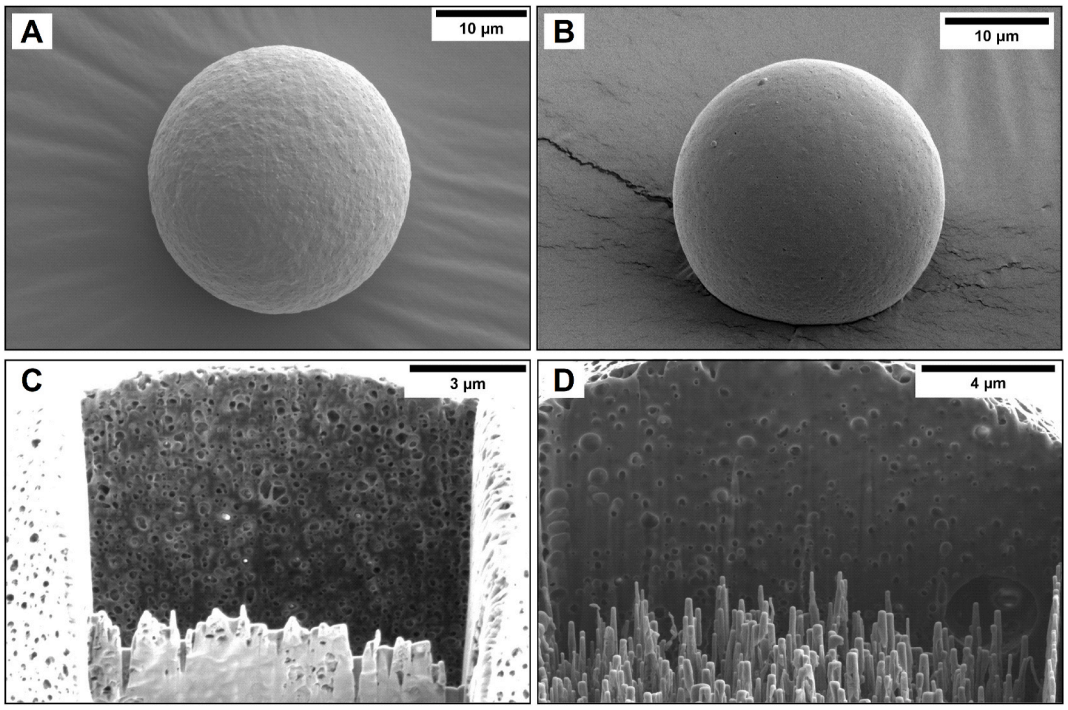

Fig. 4. Electron microscopy analysis of BUP-Depot particles. (A) SEM image of a BUP-Depot microparticle before irradiation. B) BUP-Depot microparticle after irradiation. (C) Focused ion beam analysis (FIB-SEM) of a BUP-Depot microparticle before and (D) after irradiation.

particularly with regard to particle properties, indicate a robust and highly reproducible preparation method.

Product shelf-life depends on several parameters like storage temperature, glass transition temperature (Tg) of the formulation, and residual moisture content of the final product. Long-term stability of PLGA formulations is dependent on temperature, as storage conditions have to be above the glass transition temperature of the product at all times. However, Tg of PLGA (50:50) is low and ranges between 33 and 50 °C [51,52]. Additionally, residual moisture levels after lyophilization need to be monitored carefully, as water exhibits a plasticizing effect on PLGA, lowering Tg even further [27]. High residual moisture levels promote the risk of premature polymer degradation, altering release and molecular weight, and limiting safety and use of reconstituted formulations [26–28]. In our study, the residual moisture of particles was determined to be 1.2%, which is close to the desired range of ≤1% [53,54]. Long-term storage at 4 °C for six months did not change particle size, showing the stability of the formulation. Furthermore, monitoring of drug load and burst release over the same period of time, showed no significant changes. This data indicates, that the production process with a lyophilization cycle of 45 h and sucrose as a cryprotectant enables a sufficiently long shelf life at 4 °C without the loss of drug or changes in drug release profile. However, storage for a prolonged period of time at room temperature is not advisable.

BUP-Depot was primarily developed for pain management in laboratory rodents. Injection of pain medication during experiments is therefore done over several hours. Hence, evaluation of the shelf life of a reconstituted formulation is essential. Our studies showed that BUP-Depot can be stored at least 24 h at 4 °C after reconstitution with sterile saline solution. The subsequent release kinetics remained unchanged. Such formualation stability should result in an unaltered pharmacokinetic profile and analgesic effect.

Parenteral applications must meet pharmaceutical quality standards like sterility and the absence of endotoxins [29,50,55]. Microparticle preparation is a multi-step process, with different phases like particle formation, solvent evaporation, filtration, centrifugation and lyophilization. Working under aseptic conditions can therefore be a challenge. Even without special attention to aseptic conditions, the endotoxin levels of BUP-Depot (0.12 EU/mL) were considerably lower than the required 5 EU/mL defined by the European Pharmacopeia 10.0 [50]. Product sterility is guaranteed by terminal sterilization. In the pharmaceutical industry, gamma and electron beam irradiation for drug sterilization are approved techniques [29]. If the initial bio burden is not known, an irradiation dose of 25 kGy is generally accepted [29]. Several studies have shown that irradiation leads to dose-dependent decrease in molecular weight of PLGA particles, altered drug release, changes in morphology, and an increase in particle size [30,32,34]. Therefore, the evaluation of particle sensitivity based on different PLGA types and particle sizes to irradiation was considered to be an important task. Sterilization in our study was performed by x-rays. Although this is not a standard procedure, primary interaction with matter is mediated by photons and similar to gamma irradiation. Indeed, Croonenborghs et al. [56] showed that both types of radiation had similar effects on polymers (e.g. polyethylene, polypropylene, polystyrene, polyvinylchloride). Irradiation with a total dose of 30.4–30.8 kGy resulted in insignificant changes for BUP-Depot regarding size, morphology, burst, release pattern, and cumulative release within 72 h. Only drug load was significantly reduced by 24% ($P = 0.025$), due to the degradation of buprenorphine. HPLC chromatograms of irradiated samples confirmed this hypothesis based on the appearance of additional peaks (data not shown). With respect to the size of individual particles, no significant changes were observed. However, irradiation seems to induce particle agglomeration. Similar findings were reported previously [30]. In summary, the influence of x-rays on different PLGA formulations was small. But, surprisingly, encapsulated buprenorphine showed high sensitivity towards x-ray radiation.

Pilot experiments suggest that the production of drug-loaded particles can be scaled to an industrial level by introducing a mass transfer process, i.e., solvent removal through evaporation (see section “Scale-up” in chapter 2.2). The particles formed during the first production stage are thereby transferred into a rotary evaporator, where under reduced pressure (699 mbar), the dichloromethane is removed from the solution. At this stage, the separation of the particles from the buffer

solution is performed by vacuum-assisted filtration. The product is then washed off the filter by a sucrose solution and is transferred into lyophilization vials. In this setup, filtration is the only critical unit operation. There are several issues with the filtration, mainly connected with the filter clogging. The further application of an evaporation process allows avoiding the filtration step entirely. In this case, the rotary evaporation continues after the DCM removal stage, under lower pressure (300 mbar), until the water level drops below the volume required for bottling into lyophilization vials. The remaining liquid with concentrated particles is topped up with sucrose solution and subjected to a lyophilization cycle of 45 h. The proposed method allows for the preparation of larger quantities and excludes API loss during the washing and decanting steps. It should be noted that the rotary evaporation process is a commonly used technique for production on a kilogram scale. Furthermore, there are well-established methods to scale the mass transfer processes, such as evaporation, for industrial-scale manufacturing.

5. Conclusion

Our novel buprenorphine depot formulation, based on PLGA microparticles and produced by a robust and highly reproducible method, can be stored for at least 6 months as a lyophilisate and can be used for at least 24 h after reconstitution. Pilot experiments suggest that scale-up and future aseptic production should be feasible. Although x-rays showed no influence on PLGA based microparticle morphology and performance, the encapsulated drug appeared to be sensitive to this type of irradiation. Consequently, attention should be given to aseptic manufacturing protocols. We conclude from the present study that BUP-Depot shows promising attributes with respect to future industrial production and commercialization.

CRediT authorship contribution statement

Viktoria Schreiner: Methodology, Investigation, Writing – original draft. **Pascal Detampel:** Supervision, Writing – review & editing. **Paulin Jirkof:** Validation, Writing – review & editing. **Maxim Puchkov:** Methodology, Investigation, Writing – review & editing. **Jörg Huwyler:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank Evi Bieler and Daniel Mathys from Nano Imaging Lab, SNI, University of Basel for the support for scanning electron microscopy. The authors thank furthermore Dr. Arel Su (F. Hoffmann-La Roche Ltd, Basel) and Dr. Elvire Fournier (Idorsia Pharmaceuticals Ltd, Basel) for their support as well as the many colleagues from industry and regulatory authorities for their feedback and support. We thank Dr. Maryam Farzan for her help with FIB-SEM imaging. Special thanks to Darryl Borland for his expert technical support. Financial support for the project was obtained by grants from Unitectra (University of Basel), University of Zürich, Interpharma Basel, F. Hoffmann-La Roche Ltd Basel, and Novartis Ltd Basel.

References

[1] W.H. Helfand, D.L. Cowen, Evolution of pharmaceutical oral dosage forms, Pharm. Hist. 25 (1) (1983) 3–18.
[2] A.S. Hoffman, The origins and evolution of “controlled” drug delivery systems, J. Contr. Release 132 (3) (2008 Dec 18) 153–163.
[3] Emil SE, Albert PR. Surgical sutures [Internet]. 1963 [cited 2021 Apr 18]. Available from:: https://patents.google.com/patent/US3297033A/en.
[4] A. Jain, K.R. Kunduru, A. Basu, B. Mizrahi, A.J. Domb, W. Khan, Injectable formulations of poly(lactic acid) and its copolymers in clinical use, Adv. Drug Deliv. Rev. 107 (2016 Dec 15) 213–227.
[5] K. Park, S. Skidmore, J. Hadar, J. Garner, H. Park, A. Otte, et al., Injectable, long-acting PLGA formulations: analyzing PLGA and understanding microparticle formation, J. Contr. Release 304 (2019 Jun 28) 125–134.
[6] Buprenorphine extenden-release injection for subcutaneous use. [Internet]. [cited 2021 Apr 18]. Available from:: https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/209819s000lbl.pdf.
[7] Leuprolide acetate for injection [Internet]. [cited 2021 Apr 18]. Available from:: https://www.accessdata.fda.gov/rugsatfda_docs/nda/2016/021343Orig1s033.pdf.
[8] Somatropin injectable depot suspension. [Internet]. [cited 2021 Apr 18]. Available from:: https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/21075s008lbl.pdf.
[9] Risperidone long-acting injection. [Internet]. [cited 2021 Apr 18]. Available from:: https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021346_s31_s35_s38_s39lbl.pdf.
[10] M. Vert, J. Mauduit, S. Li, Biodegradation of PLA/GA polymers: increasing complexity, Biomaterials 15 (15) (1994 Dec 1) 1209–1213.
[11] J.M. Anderson, M.S. Shive, Biodegradation and biocompatibility of PLA and PLGA microspheres, Adv. Drug Deliv. Rev. 64 (2012 Dec 1) 72–82.
[12] E.M. Elmowafy, M. Tiboni, M.E. Soliman, Biocompatibility, biodegradation and biomedical applications of poly(lactic acid)/poly(lactic-co-glycolic acid) micro and nanoparticles, J Pharm Investig 49 (4) (2019 Jul 1) 347–380.
[13] R.E. Eliaz, D. Wallach, J. Kost, Delivery of soluble tumor necrosis factor receptor from in-situ forming PLGA implants: in-vivo, Pharm. Res. (N. Y.) 17 (12) (2000 Dec) 1546–1550.
[14] M. Parent, C. Nouvel, M. Koerber, A. Sapin, P. Maincent, A. Boudier, PLGA in situ implants formed by phase inversion: critical physicochemical parameters to modulate drug release, J. Contr. Release 172 (1) (2013 Nov 28) 292–304.
[15] P.N.R. Nair, J. Schug, Observations on healing of human tooth extraction sockets implanted with bioabsorbable polylactic-polyglycolic acids (PLGA) copolymer root replicas: a clinical, radiographic, and histologic follow-up report of 8 cases, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 97 (5) (2004 May 1) 559–569.
[16] A. Budhian, S.J. Siegel, K.I. Winey, Production of haloperidol-loaded PLGA nanoparticles for extended controlled drug release of haloperidol, J. Microencapsul. 22 (7) (2005 Jan 1) 773–785.
[17] P. Strack, R. Külzer, F. Sommer, T. Bretschneider, O.M. Merkel, A. Grube, A smart approach to enable preclinical studies in pharmaceutical industry: PLGA-based extended release formulation platform for subcutaneous applications, Drug Dev. Ind. Pharm. 46 (4) (2020 Apr 2) 635–645.
[18] C.-H. Lee, K.-C. Hung, M.-J. Hsieh, S.-H. Chang, J.-H. Juang, I.-C. Hsieh, et al., Core-shell insulin-loaded nanofibrous scaffolds for repairing diabetic wounds, Nanomed. Nanotechnol. Biol. Med. 24 (2020 Feb 1), 102123.
[19] P. Perugini, I. Genta, B. Conti, T. Modena, F. Pavanetto, Periodontal delivery of ipriflavone: new chitosan/PLGA film delivery system for a lipophilic drug, Int. J. Pharm. 252 (1) (2003 Feb 18) 1–9.
[20] C.S.W. Chong, M. Cao, W.W. Wong, K.P. Fischer, W.R. Addison, G.S. Kwon, et al., Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery, J. Contr. Release 102 (1) (2005 Jan 20) 85–99.
[21] S.K. Hunter, M.E. Andracki, A.M. Krieg, Biodegradable microspheres containing group B Streptococcus vaccine: immune response in mice, Am. J. Obstet. Gynecol. 185 (5) (2001 Nov 1) 1174–1179.
[22] A. Brown, S. Zaky, H. Ray, C. Sfeir, Porous magnesium/PLGA composite scaffolds for enhanced bone regeneration following tooth extraction, Acta Biomater. 11 (2015 Jan 1) 543–553.
[23] I. Kim, H.J. Byeon, T.H. Kim, E.S. Lee, K.T. Oh, B.S. Shin, et al., Doxorubicin-loaded highly porous large PLGA microparticles as a sustained- release inhalation system for the treatment of metastatic lung cancer, Biomaterials 33 (22) (2012 Aug 1) 5574–5583.
[24] H. Kamali, E. Khodaverdi, F. Hadizadeh, S.A. Mohajeri, In-vitro, ex-vivo, and in-vivo evaluation of buprenorphine HCl release from an in situ forming gel of PLGA-PEG-PLGA using N-methyl-2-pyrrolidone as solvent, Mater. Sci. Eng. C 96 (2019 Mar 1) 561–575.
[25] A. Schoubben, M. Ricci, S. Giovagnoli, Meeting the unmet: from traditional to cutting-edge techniques for poly lactide and poly lactide-co-glycolide microparticle manufacturing, J Pharm Investig 49 (4) (2019 Jul 1) 381–404.
[26] Y. Wang, D.J. Burgess, Influence of storage temperature and moisture on the performance of microsphere/hydrogel composites, Int. J. Pharm. 454 (1) (2013 Sep 15) 310–315.
[27] P. Blasi, S.S. D'Souza, F. Selmin, P.P. DeLuca, Plasticizing effect of water on poly (lactide-co-glycolide), J. Contr. Release 108 (1) (2005 Nov 2) 1–9.
[28] M. Weert van de, W.E. Hennink, W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles, Pharm. Res. (N. Y.) 17 (10) (2000 Oct 1) 1159–1167.
[29] European Pharmacopoeia, 5.1.1, Methods of preparation of sterile products, in: European Pharmacopoeia 8, zero ed., 2014. European directorate for the quality of medicines & health care.
[30] S. Çalış, S. Bozdağ, H.S. Kaş, M. Tunçay, A.A. Hıncal, Influence of irradiation sterilization on poly(lactide-co-glycolide) microspheres containing anti-inflammatory drugs, Il Farmaco 57 (1) (2002 Jan 1) 55–62.

[31] A. Gèze, M.C. Venier-Julienne, J. Cottin, N. Faisant, J.P. Benoit, PLGA microsphere bioburden evaluation for radiosterilization dose selection, J. Microencapsul. 18 (5) (2001 Oct) 627–636.
[32] A.G. Hausberger, R.A. Kenley, P.P. DeLuca, Gamma irradiation effects on molecular weight and in vitro degradation of poly(D,L-Lactide- CO -glycolide) microparticles, Pharm. Res. (N. Y.) 12 (6) (1995 Jun 1) 851–856.
[33] G. Spenlehauer, M. Vert, J.P. Benoit, A. Boddaert, In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method, Biomaterials 10 (8) (1989 Oct 1) 557–563.
[34] S.C.J. Loo, C.P. Ooi, Y.C.F. Boey, Radiation effects on poly(lactide-co-glycolide) (PLGA) and poly(l-lactide) (PLLA), Polym. Degrad. Stabil. 83 (2) (2004 Feb 1) 259–265.
[35] B. Foster, R. Twycross, M. Mihalyo, A. Wilcock, Buprenorphine, J. Pain Symptom Manag. 45 (5) (2013 May 1) 939–949.
[36] P. Marquet, Pharmacology of high-dose buprenorphine, in: Buprenorphine Therapy of Opiate Addiction, Humana Press, Totowa, NJ, 2002 [cited 2021 Apr 18]. pp. 1–11. (Forensic Science and Medicine). Available from: https://link.springer.com/chapter/10.1007/978-1-59259-282-1_1.
[37] A. Cowan, Buprenorphine: the basic pharmacology revisited, J. Addiction Med. 1 (2) (2007 Jun) 68–72.
[38] J.V. Roughan, P.A. Flecknell, Buprenorphine: a reappraisal of its antinociceptive effects and therapeutic use in alleviating post-operative pain in animals, Lab. Anim 36 (3) (2002 Jul 1) 322–343.
[39] S. Gopal, T.-B. Tzeng, A. Cowan, Characterization of the pharmacokinetics of buprenorphine and norbuprenorphine in rats after intravenous bolus administration of buprenorphine, Eur. J. Pharmaceut. Sci. 15 (3) (2002 Apr 1) 287–293.
[40] M. Ohtani, H. Kotaki, K. Uchino, Y. Sawada, T. Iga, Pharmacokinetic analysis of enterohepatic circulation of buprenorphine and its active metabolite, norbuprenorphine, in rats, Drug Metab. Dispos. 22 (1) (1994 Feb) 2–7.
[41] S. Yu, X. Zhang, Y. Sun, Y. Peng, J. Johnson, T. Mandrell, et al., Pharmacokinetics of buprenorphine after intravenous administration in the mouse, JAALAS 45 (3) (2006 May;1) 12–16.
[42] N.M. Gades, P.J. Danneman, S.K. Wixson, E.A. Tolley, The magnitude and duration of the analgesic effect of morphine, butorphanol, and buprenorphine in rats and mice, Contemp. Top. Lab. Anim. Sci. 39 (2) (2000 Mar) 8–13.
[43] M.K. Meijer, B.M. Spruijt, L.F.M. Zutphen van, V. Baumans, Effect of restraint and injection methods on heart rate and body temperature in mice [Internet]. [cited 2021 Apr 18]. Available from: https://journals.sagepub.com/doi/abs/10.1258/002367706778476370, 2006.
[44] M. Guarnieri, C. Brayton, B.M. Tyler, A long-term study of a lipid-buprenorphine implant in rats, J. Vet. Med. 2018 (2018), 2616152.
[45] M. Guarnieri, C. Brayton, R. Sarabia-Estrada, B. Tyler, P. McKnight, L. DeTolla, Subcutaneous implants of a cholesterol-triglyceride-buprenorphine suspension in rats, J. Vet. Med. 2017 (2017), 3102567.
[46] P.L. Foley, H. Liang, A.R. Crichlow, Evaluation of a sustained-release formulation of buprenorphine for analgesia in rats, J Am Assoc Lab Anim Sci 50 (2) (2011 Mar) 198–204.
[47] E.T. Carbone, K.E. Lindstrom, S. Diep, L. Carbone, Duration of action of sustained-release buprenorphine in 2 strains of mice, JAALAS 51 (6) (2021 Nov) 815–819, 15.
[48] V. Schreiner, M. Durst, M. Arras, P. Detampel, P. Jirkof, J. Huwyler, Design and in vivo evaluation of a microparticulate depot formulation of buprenorphine for veterinary use, Sci. Rep. 10 (1) (2020 Oct 14) 1–14.
[49] M. Farzan, R. Roth, G. Québatte, J. Schoelkopf, J. Huwyler, M. Puchkov, Loading of porous functionalized calcium carbonate microparticles: distribution analysis with focused ion beam electron microscopy and mercury porosimetry, Pharmaceutics 11 (1) (2019 Jan) 32.
[50] European Pharmacopoeia, 5.1.10, Guidelines for using the test of bacterial endotoxins, in: European Pharmacopoeia 8, zero ed., 2014. European directorate for the quality of medicines & health care.
[51] Predictors of glass transition in the biodegradable poly-lactide and poly-lactide-co-glycolide polymers, in: P. In Pyo Park, S. Jonnalagadda (Eds.), J. Appl. Polym. Sci. 100 (3) (2006 May 5) 1983–1987.
[52] J.C. Middleton, A.J. Tipton, Synthetic biodegradable polymers as orthopedic devices, Biomaterials 21 (23) (2000 Dec 1) 2335–2346.
[53] N.A. Williams, G.P. Polli, The lyophilization of pharmaceuticals: a literature review, PDA J. Pharm. Sci. Technol. 38 (2) (1984 Mar 1) 48–60.
[54] J.F. Carpenter, M.J. Pikal, B.S. Chang, T.W. Randolph, Rational design of stable lyophilized protein formulations: some practical advice, Pharm. Res. (N. Y.) 14 (8) (1997 Aug;1) 969–975.
[55] Niazi S. Handbook of Pharmaceutical Manufacturing Formulations, Second Edition [Internet]. CRC Press; [cited 2021 Apr 18]. Available from:: http://www.crcnetbase.com/doi/book/10.1201/b14437.
[56] B. Croonenborghs, M.A. Smith, P. Strain, X-ray versus gamma irradiation effects on polymers, Radiat. Phys. Chem. 76 (11) (2007 Nov 1) 1676–1678.

4. DISCUSSION

Sufficient pain-relief is a prerequisite for biomedical research, as it contributes to animal welfare and avoids stress-induced artefacts. Moderate to severe pain after surgical interventions is commonly treated by subcutaneous injection of buprenorphine. However, its short terminal half-life leads to limited duration of action, necessitating several administrations per day to achieve effective analgesia. Therefore, the aim of this project was to develop a safe and effective buprenorphine depot formulation, to prolong analgesic effect in rodents.

As a platform for controlled release, PLGA based microparticles were chosen, characterized *in vitro* and tested *in vivo*.

1.7 PLGA Based Buprenorphine Loaded Microparticles

BUP's high analgesic potency coupled with the low risk of severe side effects like respiratory depression, make it a highly valuable drug [34,94,140]. So far, only immediate acting formulations are available on the European market, which need to be injected every 6-12 hours depending on the severity of pain [60,64,96,104,135,162,219]. To improve animal welfare, alternative routes of BUP administration were presented and discussed in the last years. BUP was added to eatables or drinking water to avoid recurring injections and animal handling [33,58,69,80,96,104,197,215,218]. However, surgeries, circadian rhythm but also opioids themselves, tend to influence water and food intake [54,58,87,121,197]. Therefore, these methods have been viewed as controversial. Moreover, it is difficult to control the ingested amount of BUP in laced water or feed, leading to suboptimal plasma levels or even overdoses in individual animals [54,82,197]. A further controversial topic is the oral dose required to achieve reliable analgesia. Doses between 0.5-10 mg/kg of BUP are proposed and described as effective [33,58,68,123,135,218,219]. Higher doses however pose a problem, since BUP's seemingly bitter taste limits palatability, which is a prerequisite to voluntary ingestion [219]. A further alternative route of administration for veterinary use, are transdermal

BUP patches. Once applied, they provide controlled release of BUP for several days [4,156,179]. However, animals like rodents cannot profit from this approach due to their small body size, as patch sizes are approximately 50 cm^2 [67]. Furthermore, patches need to be secured through bandages, otherwise risk of loss or even ingestion by animals is high [4,158,216]. Even though duration of action was shown to be significantly prolonged in comparison to standard single subcutaneous injections, onset of analgesic effect is late and poses a big disadvantage [4,156,179]. Therefore, development of a formulation for a single injection shortly before or during surgery with subsequent analgesic effect for several days would be the most advantageous.

Products with a similar idea were introduced to the US market in the last years. In-situ forming implant Buprenorphine SR-Lab® and Animalgesics for Mice® [60,70,71,139]. Both formulations have been studied in different animals and proved to be effective in alleviating pain [12,24–26,60,70,73,101,157,163,164,169,198,205,222,229]. Despite the advantages of both formulations, Buprenorphine SR-Lab® presented several problems including side effects at injection site (*e.g.* scabbing, erythema, necrosis) and difficulties regarding handling. Studies showed, that the high viscosity of the injection solution impeded accurate aspiration and injection [24–26,60,157,201,205]. Animalgesics for Mice® is injected with a 32 times higher dose in mice compared to standard immediate acting solutions, raising questions regarding side effects and mechanism of controlled release [222]. Furthermore, import of both products to Europe is so far not possible.

To overcome the disadvantages of the current available sustained-release formulations, an injectable microparticulate BUP depot formulation with fast onset of action, prolonged analgesic effect, easy handling and absence of side effects was designed during the course of this project. PLGA, as one of the most studied polymers for extended release, was used as a matrix, since its biocompatible and biodegradable characteristics make it an excellent vehicle [5,47,132]. Typically, PLGA based formulations can attain controlled release ranging from weeks to several months, as can be seen in table 6. The highly customizable release profile due to the polymer type is a substantial advantage for development.

Buprenorphine is commonly used for post-surgical pain alleviation for up to three days. Hence, duration of action of a novel formulation should cover a similar period of time

after single injection. PLGA with a composition of 50:50 PLA/PGA was chosen for the depot formulation, since this ratio shows a fast degradation [132,143,149]. Furthermore, molecular weight of utilized polymers was low, as this facilitates rapid drug release [92,93,150]. This approach was supported by the findings, that formulation RG 502 H-Big released 87% of drug within 3 days, while formulation RG 503 H-Big with twice the molecular weight, released only 50% during that time. *In vitro* release of BUP out of microparticles produced by polymers with terminal alkyl esters or acidic groups was also evaluated. As expected, particles prepared with polymers exhibiting ester groups showed slower drug release. This can be explained through the less hydrophilic properties of these polymers as well as the absence of autocatalytic influence of acidic groups [221,233]. Formulation RG 502 displayed for this reason only 37% release within 3 days, compared to 87% of release for acidic end group polymers.

Control of release can be achieved through the choice of polymer but also through morphology of formulation. Microparticulate formulations were selected as the most suitable type, as size and morphology of particles can assist customization of release kinetics [132]. Furthermore, microparticles are stored commonly as lyophilized powders, increasing the shelf life of final product [180]. Additionally, handling of microparticulate suspensions is easier, as in-situ forming implant formulations display high viscosities, making accurate aspiration and injection of small volumes for mice and rats demanding [24,201]. Furthermore, suspensions allow for smaller needle sizes with less pain, especially important for small animals like rodents [22,248]. Characterization of produced microparticles confirmed, that release of drug out of microparticle is size dependent. Smaller particles based on same PLGA type released drug faster compared to bigger ones. Here, complete release of BUP was reached within 72 hours (RG 502 H-Small), while larger particles showed only 87% release during that time (RG 502 H-Big). This can be explained by the greater surface area of smaller particles and the smaller diffusion pathways for BUP. Morphology of formulation RG 502 H-Big supported the theory, that release of drug is especially governed by drug diffusion through pores, as cross sections of particles prepared by focused ion beam revealed a highly porous inner structure.

Burst release of drug is often described as an unwanted phenomenon and can be attributed to the drug being encapsulated on or close to the particle surface [173]. However, since BUP is given shortly before or during surgery as pre-emptive analgesia, burst release is even desired to enable fast onset of action. Therefore, the lead formulation was identified as particles, which released 30% of drug within the first hour. Furthermore, *in vitro* release of 80-90% within 3 days was considered an optimal release profile, resulting in selection of formulation RG 502 H-Big (BUP-Depot) as most promising product.

Microparticles in this study were produced by the solvent evaporation/extraction method as described in chapter 1.6.1 and displayed in figure 3. This represents the simplest and most used preparation technique [199]. Produced particles for BUP-Depot exhibited average sizes from 20-67 µm. Broad size distributions with limited reproducibility are regularly listed as major drawbacks for this particular preparation technique. A more advanced approach to produce mono disperse particles would be the membrane emulsification and solvent evaporation/extraction method. It was shown, that particle size could be accurately controlled and reproduced by this technique [199]. Although shortcomings of this method included membrane fouling and low productivity, membrane emulsification could be used to prepare more size uniform particles with better reproducibility. However, it remains to be seen if monodispersed particles are advantageous and if reproducibility will be a concern during scale up with the current conventional production method.

1.8 Pharmacokinetic and Pharmacodynamic Studies in Mice

To assess performance of the novel depot formulation, *in vivo* studies were performed in female C57BL/6J mice. The choice to restrict experiments to the female sex was based on considerations regarding limitation of animal numbers. BUP is a well studied drug and effectiveness was shown in both male and female mice [30,78,155]. However, recent studies demonstrated, that pain transduction and pain response differ significantly depending on sex [151,206,207]. Furthermore, it was shown that analgesic action is depending on animal strain and genetics. Nevertheless, it is not

possible to test a novel drug formulation on all known rodent strains as well as both sexes. To reduce animal numbers, one strain has to be chosen to provide reliable data on analgesic effect. Mogil and colleagues demonstrated in their study of nociceptive responses of different mouse strains, that C57BL/6J mice were sensitive responders in the hotplate assay [154]. It is to be expected, that this strain will show even small deviations in analgesic action. Moreover, female C57BL/6J mice were chosen, as group-housed males tend to show aggression, resulting in stress and physical wounds leading to variability in results [40]. Nevertheless, future analgesic studies with mice need to include male animals and findings need to be confirmed in other strains.

To characterize analgesic effect accurately, several *in vivo* evaluations were done in this work. First, exposure of BUP after single subcutaneous injection of depot formulation was determined in plasma and brain tissue. Pharmacokinetic analysis is an easy way to predict effect through correlation with pharmacodynamics. It was proposed, that plasma concentrations of 1 ng/ml provide sufficient analgesia in humans and rats [59]. Therefore, plasma levels are commonly compared to this value to anticipate magnitude and duration of effect. However, not many studies correlate plasma levels and effect in animals. Ohtani et al. showed that correlation of BUP in plasma to analgesic effect measured by analgesiometric tests is poor [167]. Similar findings were made by Koltzenburg et al. in humans [112]. Furthermore, Ohtani and colleagues demonstrated that a good correlation could be obtained from specific binding concentrations in the brain and analgesic effect. This can be easily explained, since the target site of opioids for analgesia are the receptors in the brain. This thesis takes up the uncertainty regarding therapeutic threshold by correlating plasma and brain levels to analgesic effect.

Plasma levels of BUP-Depot after single injection of 1.2 mg/kg stayed above the threshold of 1 ng/ml for 12 hours, while specific binding concentrations in brain remained above the threshold for the whole duration of 72 hours. Assessment of analgesic effect after single subcutaneous administration showed significantly increased withdrawal latencies during the hotplate assay for at least 24 hours. After 48 hours latencies were still increased compared to previous control groups, however results were not significant. Although plasma levels 24 hours post injection of BUP-Depot were clearly below 1 ng/ml, definite analgesic effect could be observed.

Even though effect of non-retard formulation was not significant 12 hours post injection, latencies were increased. Interestingly, plasma levels at this time point for immediate acting BUP were similar to those 24 hours post injection of BUP-Depot, namely 0.4 ng/ml. However, significant increase in withdrawal latencies was only seen for retard formulation. This suggests that a correlation of plasma levels to analgesic action is rather poor and other factors need to be taken into account to explain these results. Comparison of specific binding concentrations of BUP in the brain after single injection of non-retard formulation and BUP-Depot show differences that are more distinct at the same time points. Non-retard formulation resulted in concentrations of 3.2 ng/g 12 hours post injection while BUP-Depot led to brain concentration levels of 5.4 ng/g 24 hours post injection, even though plasma levels at those time points were similar. Increased brain exposure can be explained by accumulation of lipophilic BUP in brain tissue and the often-described slow receptor dissociation [17,241]. Even though both concentrations are above 1 ng/g, only BUP-Depot shows analgesic effect in the hotplate assay. Therefore, it can be speculated that specific binding concentrations in the brain need to be definite above 3 ng/g to produce an effect. From the obtained data, it is evident that correlation of brain exposure to analgesic effect is much better compared to correlation of plasma levels to effect. Furthermore, our studies confirmed that duration of effect of non-retard formulations is less than 12 hours.

While 5 out of 7 animals had no measurable BUP concentrations 24 hours after non-retard formulation, only one mouse displayed levels below the limit of quantification in the BUP-Depot group 72 hours post-injection. Future studies will need to evaluate how long BUP is released out of microparticles *in vivo* and at what time point no BUP is found in plasma or brain tissue. Likewise, nociceptive tests have to be conducted in future studies for at least 72 hours to establish a time point after which withdrawal latencies return to baseline values.

Recommendations with respect to use of BUP for post-surgical pain relief based solely on pharmacokinetic studies and analgesiometric tests are controversial. As described previously, the threshold of the drug in plasma above which an analgesic effect can be expected is not assuredly established. Although many studies measure BUP concentrations in blood, not much work is presented correlating plasma exposure and

analgesic action. Furthermore, while analgesiometric tests measure solely nociceptive threshold, post-operative pain is mediated through different neurological mechanisms and can be therefore not compared [116,188]. Consequently, novel analgesic drugs and drug formulations have to be tested in a clinical setting. To meet these needs, BUP-Depot was assessed additionally after one-side sham embryo transfer. It was shown, that this comparably small surgery causes moderate pain for less than 24 hours [8,191]. To appraise pain and pain alleviation of mice after single injection of BUP-Depot, different tools were applied. Pain was measured directly by the MGS and indirectly through the assessment of clinical parameters like body weight, food and water intake as well as nesting behavior. Due to ethical considerations, the use of control animals without post-surgical analgesia was avoided. Therefore, the control group received injections of non-retard BUP every 6 hours as a common pain alleviation protocol. Overall, results suggest that both formulations successfully alleviated pain after one injection of the depot formulation or several injections of non-retard formulation. No impairments were observed as indicated by absence of clinical relevant body weight reduction, reduction in food or water intake or decreased nesting behavior. Interestingly, even though parameters were not decreased by clinically relevant levels, both groups showed reduced body weight (less than 5%) and food intake post-surgery. Food intake for both groups was reduced by approximately 50% for 24 hours post-surgery, but was back to baseline levels after an additional 24 hours. Since common pain treatment protocols for a non-retarded formulation were used, it can be assumed that pain alleviation was achieved. Therefore, the suitability of clinical parameters like body weight, food and water intake to assess general wellbeing of animals is questionable, as was already shown by other studies. It is commonly known, that decreased intake of feed or water can stem from opioids, as these are regularly described side effects [87,121]. In a study of Kendall and colleagues, mice showed decreased body weight after laparotomy throughout the 72 hours observation period, regardless if analgesic treatment was provided or not. The authors concluded, that reduced weight might not be an effective parameter to evaluate analgesic efficacy [109].

Nesting behavior is a spontaneous, species-specific action that has been proposed as a valuable indicator to assess animal welfare in rodents [99]. Changed nest building

performance can be attributed to diseases and neurological impairments [35,39,41,42,57,119]. However, Jirkof et al. demonstrated that nest complexity scores were reduced in male and female mice after sevoflurane anesthesia without any surgical intervention. Furthermore, sham embryo transfer led to even lower scores regardless of applied or omitted analgesic treatment. Jirkof and colleagues concluded, that nest complexity scores are useful to assess reduced wellbeing, but determination of efficacy of given pain treatment is not possible [99]. Nest complexity scores in this study were higher 24 hours post-surgery as compared to baseline measurements, meaning that mice build and improved their nests after anesthesia and surgery. Even if assessment of efficacy of a given analgesic is not possible through this test, it is evident that wellbeing of mice was not reduced, which can be attributed to the given pain treatment.

In recent years, a novel tool to appraise pain in mice and rats was introduced. The MGS or RGS is used to quantify spontaneous pain according to objective scoring of facial expressions [115]. Successful use of MGS and RGS was demonstrated in several studies to recognize pain and insufficient pain treatment [115,138]. However, it must be taken into account that the score shows sensitivity towards sex, strain but also to applied anesthesia [81,144,145,148]. Measured MGS scores for BUP-Depot and non-retard formulation group were low 3-12 hours post-surgery, indicating efficacy of pain treatment. Furthermore, the decrease of the score to almost zero 12 hours after the sham ovariectomy, confirms the short duration of pain caused by this type of surgery. A limitation of the action unit “whisker change” was observed in this work, as mice tended to smear the chamber glass with eye ointment, making it impossible to evaluate the change in whisker position properly. Nevertheless, the MGS presents an easy, reliable, sensitive and objective pain evaluation tool [138].

Pain assessment in rodents is difficult, and many different methods were proposed in recent decades. Every method has advantages and disadvantages and is influenced by different factors. To assess pain reliably in animals, a multitude of parameters have to be evaluated to balance the drawbacks.

The aim of this work was furthermore to evaluate side effects and handling of the novel depot formulation. Especially the case of Buprenorphine SR-Lab® from ZooPharm showed how important it is to look at the injection site, as almost all studies

investigating the in-situ forming implant formulation reported erythema, lesions or even necrosis [24–26,30,60,157,162–164,171,205,249]. After injection of BUP-Depot only two mice out of 75 demonstrated a redness of skin and a lump near or at the site of injection. It was not clear, if the formulation or other influences caused this. Nevertheless, this suggests that the proposed depot formulation is a safe alternative and presents a clear advantage compared to Buprenorphine SR-Lab®. Future studies need to evaluate if the few occurred injection site reactions were caused by the microparticulate product. Overall handling of developed BUP-Depot formulation was easy and straightforward. As the final product is supplied as a lyophilized powder in a vial, reconstitution with sterile saline solution is the only step that needs to be done by the user. Reconstitution time and viscosity are minimal since only low concentrations of sucrose are used as excipient besides microparticles. A dose of 1.2 mg/kg results in an injection volume of approximately 100 µl for a 20 g mouse, resulting in a high enough volume to comfortably work with. The big advantage of the designed formulation is the adjustable dose and injection volume, since changes in amount of reconstitution solution allow for customizable concentration of the product.

1.9 Characterization of Depot Formulation

The aim of this thesis was to design a novel depot formulation for buprenorphine with potential future commercialization. Therefore, complete characterization with respect to possible industrial production was important.

Although the first PLGA based formulation for clinical use in humans was already introduced in the 1980s, only a few formulations managed to get market access since then as can be seen in table 6 [91,239]. An often discussed problem is the physicochemical instability of microparticulate formulations due the polymer interactions with drug, solvents or other excipients [2,14,16,46,234,237]. Measurements of residual moisture levels of the novel depot formulation suggest that the low water concentration of 1.2% of the final product will not impede storage of microparticles. Furthermore, stability studies of lyophilized particles showed that no

change in particle size, drug content or burst release could be detected over a period of 6 months. Therefore, it can be expected that this product will have a sufficient long storage time.

Since the designed microparticulate formulation is intended to be used by subcutaneous injection, requirements for parenteral formulations must be met. Therefore, sterility has to be ensured and endotoxin content needs to be assessed. Our studies showed, that an endotoxin concentration of 0.12 EU/mL could be detected. This represents a more than 10 times lower dose as required by the European Pharmacopoeia (5 EU/mL) [52]. Furthermore, this thesis assessed the possibility to sterilize the final BUP loaded microparticles by X-rays. Terminal sterilization is a common way, to ensure sterility of the final product [51]. However, sensitivity of BUP towards X-rays hindered a successful application of this method. More than 20% of drug degraded during irradiation, making it a highly inefficient method. Moreover, future studies would be needed to determine if degradation products are non-toxic. It can be speculated if other sterilization techniques like electron beam or gamma irradiation would be more suitable. Therefore, more studies need to be done to assess the influence of other irradiation techniques on BUP and microparticles. Interestingly, microparticles itself did not change morphology, size or release kinetic after irradiation as can be seen in Chapter II, figure 3. The regularly presented difficulties with respect to radiation exposure of PLGA microparticles like chain scission and molecular weight reduction resulting in altered release kinetics did not occur in our studies [1,23,76,128,208].

A major concern with respect to microparticles prepared by the solvent evaporation/extraction method are the used organic solvents during preparation. Dichloromethane, which is one of the most used solvents for PLGA, belongs to Class 2 solvents, which need to be monitored and limited as described in the European Pharmacopoeia [53]. Since the preparation method used in this work is likewise utilizing dichloromethane, the residual amount of it in the final product needs to be measured. The European Pharmacopoeia is limiting the concentration of this toxic, possibly cancerogenic solvent to 600 ppm [53]. Preliminary results obtained by gas chromatography (data not shown) suggest, that residual dichloromethane did not

exceed the limits. However, future studies need to clearly establish the residual solvent concentration of the final product.

The presented results indicate that a future industrial production and commercialization is possible.

5. CONCLUSION

In conclusion, a novel, size controlled, safe and effective microparticulate PLGA depot formulation of buprenorphine was successfully developed. Analgesic effect was established through pharmacokinetic and pharmacodynamic assays. Plasma levels of BUP after single injection remained for at least 12 hours above therapeutic threshold. Specific binding concentrations of drug in the brain as the target site of opioids remained above 1 ng/ml for the whole duration of 72 hours. Furthermore, conducted nociceptive assay supported the findings through demonstration of significantly prolonged effect of BUP-Depot of at least 24 hours compared to commercial non-retard formulation with an effect of less than 12 hours. In a proof-of-concept, amelioration of post-surgical pain after one-sided sham embryo transfer was achieved after single administration reliably, as measured by clinical parameters, nesting behavior and the Mouse Grimace Scale. No notable side effects were observed, confirming safety of novel formulation.

Furthermore, characterization of BUP-Depot with respect to future commercialization showed, that the final product prepared by the established protocols allows for industrial production. Designed particles showed low residual moisture levels and stability studies at 4°C indicated sufficient shelf life. Endotoxin content of final product was approximately 10 times lower than required by European Pharmacopoeia. Terminal sterilization by X-rays revealed no influence on PLGA based microparticles. However, BUP showed high sensitive towards irradiation, which led to degradation of drug. Aseptic manufacturing is therefore required to ensure sterility of BUP-Depot. Taken together, these results indicate that the final product exhibits the right attributes for future industrial production.

The presented novel product BUP-Depot is a safe and effective formulation for prolonged pain management in laboratory mice. It can be considered as an alternative and a refinement to current analgesic protocols utilizing BUP. Due to the reduced amount of pain, stress and animal handling through fewer injections, burden for animals and caretakers can be significantly decreased.

6. OUTLOOK

For successful future industrial production and commercialization, further studies are needed with respect to scale up. It needs to be evaluated how a scale up can be realized and how it would affect reproducibility of particle size, burst release and release kinetics. In this context, further work needs to be done regarding feasibility of aseptic microparticle production, since BUP shows clear sensitivity towards X-rays as sterilization technique. Additionally, scale up studies need to investigate how a minimization of organic solvents during production is achievable. Furthermore, appropriate techniques and process parameters need to be explored to remove as much of the used solvents as possible. Amount of residual solvent in the final product needs to meet the requirements set by the European Pharmacopoeia.

In this thesis, plasma and brain exposure was evaluated for 72 hours after single injection of BUP-Depot. It could be seen, that although plasma concentrations of BUP were starting to drop below the limit of quantification after 3 days, BUP in brain was still measurable. Further experiments in mice are needed to evaluate the time point at which no drug can be detected in plasma and brain tissue. Likewise, analgesiometric studies need to be extended to assess at which time post injection, withdrawal latencies return to baseline values. Taken together, prospective research needs to establish a definite time point at which no effect of BUP is seen and no drug can be detected in the body. Moreover, pain alleviation needs to be examined in other surgical models, which produce severe pain for a longer time. In all subsequent animal studies, special attention needs to be paid to the injection site, to assess continuously the safety of the novel depot formulation.

Efficacy studies in female C57BL/6J mice demonstrated assuredly that the here developed depot formulation is able to prolong analgesic effect. However, future studies need to evaluate if the determined results apply for other mouse strains and for male animals. Moreover, studies need to be extended towards other rodents like rats but also to companion animals like cats and dogs. Especially the treatment of companion animals would broaden the field of application and increase the market potential, making this product more attractive for investors.

SUPPLEMENT

Table S1. Efficacy studies of oral and parenteral administered buprenorphine in mice and rats.

Species	Dose	Interval	Test Method	Results and Reference
Male SD Rat	0.05 mg/kg s.c. 0.4 mg/kg VI-N	PE + 8 PE + every 24 h	Catheterization, BW, WI, Corticosterone levels	**BUP s.c.:** Little impact on serum corticosterone levels. Both treatments had positive impact on BW and WI. **BUP oral:** 10 h post-surgery rats showed significant lower corticosterone levels compared to s.c. and control animals without analgesia. Afterwards no difference to s.c. [68].
Male SD & Wistar Rat	0.05 mg/kg s.c 0.4 mg/kg VI-N	PE + 8 PE + every 24 h	Catheterization, PC, BW, Corticosterone levels	**BUP s.c.:** Significant higher corticosterone levels after 2 h and 10 h. PC close to zero after 2 h. **BUP oral:** Significant lower corticosterone levels 10 h post-surgery. PC above 1 ng/ml for 2 h and close to 1 ng/ml for 14 h. **BW:** All treatments decreased weight gain in both strains compared to control without surgery. Only in Wistar rats s.c. treatment decreased weight gain significantly compared to VI [69].
Male & Female SD Rat	0.03 mg/kg s.c. 0.3-0.6 mg/kg VI-N	Once Once	TW (OPAD)	**BUP oral Male:** 0.5 and 0.6 mg/kg significant effect 1 h after ingestion. No significant difference to 0.03 mg/kg BUP s.c. **BUP oral Female:** no effect [215].
Male Wistar Rat	0.5 mg/kg VI-J	PE	Laparotomy, BW, WI, FI	**BUP oral + surgery:** Decreased effects on BW & WI after surgery compared to group receiving vehicle alone. Still, all parameters are significantly reduced even with BUP treatment compared to pre-surgery animals within group. **BUP oral + anesthesia alone:** Decrease in FI but not in BW or WI [123].

Table S1. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Female Wistar Rat	0.1-0.4 mg/kg VI-J	PE	Laparotomy	**BUP oral:** All doses prevented fall in BW and reduced depression of WI compared to control group without analgesia after surgery. FI was significantly reduced in all groups except for 0.3 mg/kg [58].
Male LE Rat	0.05 mg/kg s.c. 0.5 mg/kg VI-J 0.5-10 mg/kg OI	once once once	TW	**BUP s.c.:** Duration of action = 2 h. High doses of BUP in gelatin (≥ 0.25 mg/ml) become unpalatable. **BUP oral 0.5-2.5 mg/kg:** No effect. **BUP oral 5 mg/kg:** Duration of action = 4 h. **BUP oral 10 mg/kg:** Duration of action = 8 h [135].
Male Wistar Rat	0.1 mg/kg s.c. 0.1 mg/kg s.c + 5.6-3.9 mg/kg/d VI-W 4.8-2.4-3.2 mg/kg/d VI-WI	Once or every 8 h s.c. + drinking water for 2 d Drinking water for 3 d	TW	**BUP s.c. once:** Duration of action = 8 h. **BUP s.c. every 8 h:** No overall antinociceptive effect. WI significantly reduced on day 2 and 3 (unlaced water). **BUP oral + s.c.:** Overall significant higher withdrawal. But lasted less than 48 h despite continuous WI. **BUP oral:** Overall significant higher paw withdrawal latency compared to control. But lasted less than 72 h despite continuous WI Decline of WI after BUP treatment points to bitter taste or emesis/gastric distress. Decline of effect despite of continuous BUP points to tolerance development [96].
Male LE & SD Rat	0.05 mg/kg s.c. 0.5, 5 mg/kg OI	Once Once	TW	**BUP s.c.:** Increased threshold at 1 h. Pica was only seen in SD rats. BUP oral and s.c. induced measurable aversion to grape juice. **BUP oral 0.5 mg/kg:** No significant change in pain threshold at any time point. **BUP oral 5 mg/kg:** Increased threshold at 1 h and 4 h [219].

Table S1. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Female LE Rat	0.05 mg/k s.c. 0.5 mg/kg VI-W	Once Once	TW	**BUP s.c.:** Significant effect at 0.5 nd 1 h after injection compared to oral, vehicle alone or baseline. **BUP oral:** At the dose of 0.5 mg/kg not effective [218].
Male & Female SD Rat	0.2 mg/kg i.m. 0.6, 1.6, 2.9 mg/kg VI-W	Once Drinking water for 24 h	TW	**BUP i.m.:** Significant increase in latency 90 min after injection. **BUP oral:** Only 2.9 mg/kg/d led to significant increase in latency after 24 h. WI did not differed between BUP laced water and tap water [33].
Male NMRI Mouse	0.05 mg/kg i.v. or s.c. 0.4 mg/kg OG or VI-N	Once Once	PC	**BUP s.c. and i.v.:** Above 1 ng/ml for 6-12 h. **BUP oral:** Gavage and VI produced high serum concentrations with longer duration than s.c. and i.v. injection. Above 1 ng/ml for at least 12 h [104].
Male NMRI Mouse	0.1 mg/kg s.c. approx. 1-3 mg/kg/d VI-G	Once Once	PC	**BUP s.c.:** Plasma levels above 1 ng/ml for at least 6 h. Highly variable time point of ingestion start/ finish and highly variable amount of ingested gel. **BUP oral 1 mg/kg/d:** Plasma levels above 1 ng/ml for 6 h after 15 h of ingestion. **BUP oral 3 mg/kg/d:** Plasma levels above 1 ng/ml for 12 h after 15 h of ingestion [82].
Female CD-1 Mouse	0.1 mg/kg s.c. 0.1 mg/kg s.c. + 0.79 mg/kg/d VI-P 0.75-4.2 mg/kg/d VI-P	Once Once + Pellet for 20 h Pellet for 20 h	TW	**BUP oral:** Significant effect compared to placebo group for all oral doses and for s.c. 3 h post injection. Additionally, no differences between oral and s.c. groups. Oral dose of 0.75 mg/kg effective in this setup. No decrease in VI even at high BUP doses [155].

Table S1. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Female CD-1 Mouse	0.1 mg/kg s.c. + 0.3 mg/kg/d VI-P	PE + once for 20 h	Intra-utero Electroporation	Increase in FI combined with a lower weight loss for treatment group 24 h after surgery. Clear clinical improvement compared to vehicle only [155].
Female C57BL/6 J Mouse	0.1 mg/kg s.c. + approx. 1mg/kg/d VI-W	s.c. twice 6 h apart + Water for dark phase	One-side embryo transfer, PK, BW, FI, WI	**BUP s.c. twice + BUP oral:** BW and WI was not different to naïve mice. FI decreased significantly. 90% of mice had serum levels beneath therapeutic threshold before second s.c. in jection. Individual mice showed serum concentration at all times. **BUP s.c. every 4 h + BUP oral:** BW and WI was not different to naïve mice. FI decreased significantly. Distance moved significantly increased. Continuous serum levels for effective pain treatment. Pattern of drinking behavior was not altered after surgery. **BUP oral:** WI was generally sporadic/infrequent during light phase for all groups. BW, WI and FI were not different to naïve mice receiving only tap water [197].
	0.1 mg/kg s.c. + approx. 1mg/kg/d VI-W	s.c. twice 6 h apart + Water for dark phase		
	0.1 mg/kg s.c. + approx. 1mg/kg/d VI-W	s.c. twice 6 h apart + Water for dark phase		

s.c. = subcutaneous; i.m. = intramuscular; i.v. = intravenous;

VI-N = voluntary ingestion in Nutella; VI-P = in pellet feed; VI-W = in water; VI-J = in gelatine, VI-G = in MediGel; OI = orogastric infusion; OG = oral gavage; PE = pre-emptive; TW = thermal withdrawal; OPAD = orofacial pain assessment device WI = water intake; FI = food intake; BW = body weight; PC = plasma concentration.

Table S2. Efficacy studies of sustained-release formulations compared to non-retard formulations in animals.

Species	Dose	Interval	Test Method	Results and Reference
Male SD Rat	0.2 mg/kg s.c. NR 1.2 mg/kg s.c. SR	Once	Tibial Defect Model, TW, BW, FI, WI, Activity	**NR:** Animals showed fewer vertical raises compared to all groups throughout experiment (8 d). **SR:** Thermal latency was increased for 72 h. Significant difference to NR was found only at 48 h time point. Animals showed more vertical raises than NR animals but less compared to control without surgery. Skin irritation with erythema/scabbing at injection site. **General activity, use of affected leg, BW, FI, WI:** No significant difference between both groups.
	0.1 mg/kg s.c. NR 0.9, 1.2 mg/kg s.c. SR	Once	PC	**NR: PC** Aaove threshold for 8 h. **SR:** Both doses showed similar PC which remained above or close to the therapeutic threshold of 1 ng/ml for 72 h [60].
Male SD Rat	0.05 mg/kg s.c. NR 0.3, 1.2, 4.5 mg/kg s.c. SR	PE + twice daily for 3 d PE	Plantar Incision Model, Ethogram, TW, MW	**NR:** Effective analgesic treatment in the model for 72 h. **4.5 mg/kg SR:** BW clinically reduced and severe sedation. Mechanical and thermal postoperative withdrawal is similar for NR and SR to pre-operative baseline. **0.3 and 1.2 mg/kg SR:** Effective analgesic treatment in the model for 48-72 h [29].
Male SD Rat	0.05 mg/kg s.c. NR 1.2 mg/kg s.c. SR	PE + twice daily for 3 d PE	Plantar Incision Model, BW, TW, MW, PC	**SR and NR:** No change in BW and no difference in thermal or mechanical hypersensitivity between NR and SR after surgery and compared to baseline. Both treatments provide analgesia in this model for 96 h. Both formulations provide steady PC, but only SR shows PC above 1 ng/ml and only at 48 h time point. No significant change in plasma levels between treatment groups at any time. **SR:** highly viscous solution leading to preparation and injection problems [201].

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Male SD Rat	0.05 mg/kg s.c. NR 0.65 mg/kg s.c. ER 1.2 mg/kg s.c. SR	Once	TW, Atrial blood gas, Activity	**NR:** Activity was significantly less at 24 h time point compared to baseline for all groups. Withdrawal latency increased at 1 h. **ER:** Latency increased compared to baseline and saline from 4-48 h. Blood gas values significantly lower compared to all other groups. **SR:** Latency increased only compared to baseline at 24, 48, 72 h. But no difference to saline control animals at all times [101].
Female SD Rat	0.05, 0.1 mg/kg s.c. NR 1.2 mg/kg s.c. SR	Every 12 h for 3 d Once	Laparatomy, Ethogram, BW, PC	**0.1 mg/kg NR:** Did no provide effective analgesia, as cage-side scores were similar to animals receiving only saline solution after surgery. Hypothesis: Ceiling effect at higher BUP doses. **SR:** PC close to 1 ng/ml only at 24 h time point. Detection in plasma for at least 9 d. 80% of rats showed injection site reactions (erythematous plaques). **SR and 0.05 mg/kg NR:** BW loss in all surgery and in anesthesia only groups. No statistical analysis. All scores (ethogram) were lower compared to surgery animals without analgesic treatment [162].
Female Nude Arthymic Rat	0.6 mg/kg s.c. SR	PE	Xeno-transplantation	All rats showed nodules at injection site after 3 months. Polymer was still intact, drug has not been released. Hypothesis: Immunodeficient rodents cannot break down polymer. Usage is not recommended [171].
Fischer Rat	1.3, 3.9, 6.5 mg/kg s.c. ER	Once or on day 0, 4 and 8	Sham mini pump implant	Single or repeated treatment after surgery seem not to cause clinically significant effects for all doses [70].

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Fischer Rat	0.65, 1.35 mg/kg s.c. ER	Once	PC	**0.65 mg/kg ER**: Plasma levels above threshold for at least 48 h. Significant higher latencies for 5 d compared to control. **1.35 mg/kg ER:** Plasma levels above threshold for at least 96 h. Significant higher latencies for 5 d compared to control [70].
Male BALB/cJ & SWR/J Mouse	0.1 mg/kg s.c. NR 1.0 mg/kg s.c. SR	Once	TW	**NR:** No significant difference to control at any time point. SWR/J mice had significantly shorter baseline latencies than did BALB/cJ mice. **SR:** Significant effect at 2,6,12 h compared to control. 12 out of 15 mice developed scabby lesions, one an open wound at injection site. Viscous solution with small injection volumes was difficult to administer accurately [24].
Female CD-1 Mice	0.1 mg/kg s.c. NR 0.6 mg/kg s.c. SR	Every 12 h Once	PC	**NR:** Therapeutic plasma levels only at 2 and 48 h time point. **SR:** Therapeutic plasma levels for 24 h. Significant difference to NR at 4 and 8 h time point [108].
Male C57BL/6J Mouse	0.03, 0.05, 0.1, 2 mg/kg s.c. NR 0.3, 1.2 mg/kg s.c. SR	Once Once	PC	**0.03 and 0.05 mg/kg NR:** Failed to provide therapeutic PC. **0.1 and 2.0 NR:** Therapeutic levels for at least 3 and 6 h. **0.3 mg/kg SR:** Failed to provide therapeutic levels. **1.2 mg/kg SR:** Therapeutic PC for at least 9 h. **SR:** Several mice showed skin lesions ranging from mild dermatitis to necrosis [30].
Male Swiss-Webster Mouse	1.5 mg/kg s.c. NR 1.5 mg/kg s.c. SR	Once Once	TW, BW, RR	**NR:** BW not significantly different to control group. Significant effect in nociception tests only 4 h after injection. Respiratory rate only at 4 h significantly decreased. **SR:** Significant effect at 4, 24 and 48 h in nociception tests. Respiratory rate significantly decreased for 48 h compared to control [78].

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Male C57/BL6 Mouse	0.1 mg/kg s.c. NR	PE + every 6 h	CLP, Ethogram, Immunologic parameters	**Recommendation NR:** More frequent dosing of NR as every 6 to 12 h for first 24 h after CLP. **SR**: Clinical scores 12, 24 h postoperatively significant better compared to NR. Cytokine levels and mortality were similar for both groups [79].
	1.0 mg/kg s.c. SR	Once		
Female CD-1 Mouse	0.1 mg/kg s.c. NR	PE + every 12 h for 60 h	Laparotomy, BW, Ethogram	**NR:** No difference in wound licking to saline treated animals after surgery. Dosing every 12 h did not provide adequate analgesia, rescue analgesia would have been necessary. **SR:** Decreased wound licking at 1, 3, 6 h compared to saline and at 3, 6, 12 h compared to NR. Orbital tightening decreased at 1 h after surgery compared to saline and NR. BW decreased for all groups after surgery, therefore not useful to evaluate pain [109].
	0.6 mg/kg s.c. SR	PE		
Male & Female BALB/c Mouse	3.25, 16.25 mg/kg s.c. ER	Once or on day 0, 4 and 8	Sham mini pump implant, BW, PC	**3.25 mg/kg ER once:** Therapeutic levels were present for at least 72 h. **All doses ER:** 2-9% BW loss, no difference to control animals with surgery but without analgesic treatment. No difference in signs of pain between male and female mice and control groups. The study indicate a high tolerance to ER [222].
Male & Female BALB/c & Swiss SWR/J Mouse	0.5, 2.0 mg/kg s.c. NR	Once	TW, PC	**NR:** Withdrawal latencies showed no difference in male and female BALBc mice and control at both doses 6 h post injection. Only dose of 2 mg/kg provided PC above therapeutic threshold 6 h time point.
	0.25 mg/kg/d OS-Pump	For 5 d	Sham mini pump implant, TW	**OS-Pump:** Withdrawal latencies showed only difference at 48 h time point compared to baseline. **ER:** Only difference in latency on day 5 compared to baseline for Hargreaves test. Significant difference in tail flick test to baseline on day 1 and 2 for male BALB/c and Swiss mice [198].
	3.2 mg/kg s.c. ER	Once		

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Female Hartley Guinea Pig	0.48 mg/kg s.c. ER 0.48 mg/kg s.c. ER + carprofen	Once ER once + carprofen every 24 h	Hysterectomy, Ethogram, BW, PC, MW	**ER:** 10% or higher loss in anesthesia + ER and anesthesia + ER + surgery group compared to baseline. No difference between both treatment groups. Animals after surgery showed significant increased hypersensitivity at 32 and 96 h compared to anesthesia + ER. PC above 1 ng/ml from 24-48 h post injection after single dose. Recommendation: dosing 8-12 h before surgery. **ER + Carprofen:** Hypersensitivity increased at 2 h post injection compared to anesthesia + ER + carprofen group. Multimodal treatment provided better analgesia [169].
Female Dunkin–Hartley Guinea Pig	0.05 mg/kg s.c. NR 0.3 mg/kg s.c. SR	Every 12 h for 60 h Once	PC, MW	**NR:** PC above therapeutic threshold only at 1 h and 26 h time point. Paw withdrawal was significant different to baseline and SR at 1 h time point. Dosing twice daily does not provide sufficient analgesia. **SR:** PC above threshold from 6-26 h. Paw withdrawal different to baseline from 6-48 h, but difference to NR only at 6, 12 h. Dosing interval of 24-48 h might be appropriate. Animals developed reactions at injection site [205].
Male & Female Suffolk Sheep	0.27 mg/kg i.m, s.c. SR	Once	PC, TW	**I.m. and s.c.:** Similar pharmacokinetic. **SR:** Therapeutic PC (0.5-0.7 ng/ml) was reached after 48 h and maintained for 72 h. Effect was seen between 12 and 72 h if compared to baseline. Recommendation: Dosing 48 h prior of surgery or application of additional analgesic during the first 48 h post-surgery [229].
Female Dorset & Suffolk Sheep	0.1, 0.05 mg/kg s.c. SR	Once	PC	**0.1 mg/kg SR:** Therapeutic PC (0.1 ng/ml) from 48-192 h post injection. **0.05 mg/kg SR:** Therapeutic PC (0.1 ng/ml) from 48-72 h post injection. 2 animals showed swelling and erythema at injection site [249].

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Female Beagle Dog	0.02 mg/kg s.c. NR 0.2 mg/kg s.c. SR	PE + every 12 h, meloxicam PO daily PE, meloxicam PO daily	Ovariohysterectomy, Ethogram, PC, RR, Pain scores	One dog from each group had breakthrough pain. **NR:** PC were below threshold within 7 h. After second injection PC were maintained at 1 ng/ml for 72 h. No difference in BW or RR between SR and NR. **SR:** Significant lower pain scores (incision assessment and palatability of abdomen) at all time points. Other pain scores showed no significant difference. Therapeutic PCs were reached 15 min post injection and maintained for 72 h for both treatments. 9 out of 10 dogs had detectable BUP concentrations at 168 h time point. 7 out of 10 dogs had reactions at injection site [164].
Male and Female Mongrel Dog	0.02 mg/kg i.v. NR 0.2 mg/kg s.c. ER	Once	RR, TW, PC	**NR:** Significantly increased latency 1-12 h post injection compared to baseline. No difference in RR compared to ER. Therapeutic plasma levels for 2-4 h. **ER:** Increased latencies 1-72 h compared to baseline and at 1-60 h compared to NR. Therapeutic PC for at least 72 h [12].
Female Shorthair Cat	0.02 mg/kg OTM 0.12 mg/kg s.c. SR	PE, every 12 h PE	Ovariohysterectomy, RR, Ethogram, MW, Pain scores	No significant difference between groups with respect to RR and ethogram. Pain scores were significantly higher compared to pre-surgery values at 12 and 24 h time point in both groups. MW showed significantly higher scores during recovery from anesthesia for both groups. No change between 12-72 h between both groups and within group baseline. **SR:** One cat showed an injection site reaction [26].
Male CM & Rhesus Macaque	0.01, 0.03 mg/kg i.m. NR 0.2 mg/kg s.c. SR	Once	PC, BW	**0.01 mg/kg NR:** No effect on BW. PC above therapeutic threshold for 2 h. **0.03 mg/kg NR**: No effect on BW. Therapeutic concentration for 6 h. **SR:** No effect on BW. Therapeutic PC for more than 60 h. 40% of animals showed injection site reactions ranging from mild erythema to raised plaques [163].

Table S2. Continued.

Species	Dose	Interval	Test Method	Results and Reference
Male & Female Prairie Dog	0.9, 1.2 mg/kg s.c. SR	Once	PC	Therapeutic levels were reached between 0-4 h post injection and stayed above threshold until 96 h. Animals showed injection site reactions [25].
Male & Female Northern Elephant Seal	0.12 mg/kg s.c. SR	Once	PC	PC above 1 ng/ml was reached by 12 h. After 24 h, concentrations were below 1 ng/m for most animals. 23% of animals showed cellulitis or abscess formation between 24-162 h post injection [157].
Male & Female American Kestrel	1.8 mg/kg i.m. or s.c. SR	Once	PC	PC above therapeutic threshold for both injection site methods (i.m. and s.c.) for at least 48 h [73].

NR = non-retard Buprenorphine HCl; SR = Buprenorphine SR-Lab from ZooPharm (Fort Collins, CO); ER = Animalgesics for Mice (Millersville, MD); s.c. = subcutaneous; i.m. = intramuscular; i.v. = intravenous; OS-pump = osmotic pump; PE = pre-emptive; PO = per oral; OTM = oral transmucosal;

CM = Cynomolgus;

RR = respiratory rate; TW = thermal withdrawal latency; MW = mechanical withdrawal latency; CLP = cecal ligation and puncture model; FI = food intake; WI = water intake; BW = body weight; PC = plasma concentration

Table S3. Efficacy studies of transdermal BUP compared to parenteral BUP formulations in animals.

Species	Dose	Interval	Test Method	Results and Reference
Male Beagle Dog	70 µg/h TD	Once	PC	PC increased during 36 h post application and stayed around 0.7-1.0 ng/ml for 72 h. One dog had plasma concentrations less than 0.02 ng/ml for all time points [4].
Male & Female Beagle Dog	0.02 mg/kg i.v. NR 52.5 µg/h TD	Once Once	TW, PC	**TD:** PC 1 ng/ml was reached after 48 h and remained there until patch removal at 72 h. Effect started after 36 h and lasted until patch removal. 3 dogs showed no drug in blood. **NR:** PC above 1 ng/ml for 2 h. Effect from 20 -360 min [179].
Female Dog	0.02 mg/kg s.c. NR 70 µg/h TD	PE + every 6 h for 38 h 48 h before surgery	Ovario-hysterectomy, Pain scores, RR	Pain scores and RR were significantly lower in both groups compared to surgery without analgesia. Both formulations were equally effective to alleviate pain [156].
Female Göttingen Minipig	0.032 mg/kg i.v. NR 0.18 mg/kg s.c. SR 30 µg/h TD	Once	PC	Hypothesized therapeutic threshold: 0.1 ng/ml. **NR:** Above threshold for 8 h. **SR:** Above threshold for 264 h. 4 out of 5 pigs had injection site reactions. **TD:** Above threshold for 72 h. Delayed onset of action (12-24 h). One pig did not reach detectable plasma concentrations possibly due to hair regrowth and reduced contact area [216].
Male & Female Shorthair Cat	35 µg/h TD	Once	PC, TW, BW, FI, WI	No change in BW, FI or WI. Patch did no increase thermal latency's at any time point. Plasma levels reached 1 ng/ml only after 22 h. Recommendation: Loading dose with a 52.5 or 70 µg/h patch. Most cats lost patches due to loss of adhesiveness, possible caused by hair regrowth [158].

NR = non-retard Buprenorphine HCl; SR = Buprenorphine SR-Lab from ZooPharm (Fort Collins, CO); TD = transdermal patch; s.c. = subcutaneous; i.v. = intravenous; TW = thermal withdrawal latency; RR respiratory rate; PE = pre-emptive; WI = water intake; FI = food intake; BW = body weight; PC = plasma concentration

REFERENCES

[1] A. Gèze, M. C. Venier-Julienne, J. PLGA microsphere bioburden evaluation for radiosterilization dose selection. Journal of Microencapsulation 2001;18:627–636.

[2] Albertini B, Iraci N, Schoubben A, Giovagnoli S, Ricci M, Blasi P, Rossi C. β-cyclodextrin hinders PLGA plasticization during microparticle manufacturing. Journal of Drug Delivery Science and Technology 2015;30:375–383.

[3] Amanzio M, Pollo A, Maggi G, Benedetti F. Response variability to analgesics: a role for non-specific activation of endogenous opioids. Pain 2001;90:205–215.

[4] Andaluz A, Moll X, Ventura R, Abellán R, Fresno L, García F. Plasma buprenorphine concentrations after the application of a 70 µg/h transdermal patch in dogs. Preliminary report. Journal of Veterinary Pharmacology and Therapeutics 2009;32:503–505.

[5] Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Advanced Drug Delivery Reviews 2012;64:72–82.

[6] Andhariya JV, Choi S, Wang Y, Zou Y, Burgess DJ, Shen J. Accelerated in vitro release testing method for naltrexone loaded PLGA microspheres. International Journal of Pharmaceutics 2017;520:79–85.

[7] ARESTIN®(minocycline hydrochloride) Microspheres. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/050781s019lbl.pdf. Accessed 2 Jul 2020.

[8] Arras M, Rettich A, Cinelli P, Kasermann HP, Burki K. Assessment of post-laparotomy pain in laboratory mice by telemetric recording of heart rate and heart rate variability. BMC Vet Res 2007;3:16.

[9] Astaneh R, Erfan M, Moghimi H, Mobedi H. Changes in morphology of in situ forming PLGA implant prepared by different polymer molecular weight and its effect on release behavior. Journal of Pharmaceutical Sciences 2009;98:135–145.

[10] Authier N, Gillet J-P, Fialip J, Eschalier A, Coudore F. Description of a short-term Taxol®-induced nociceptive neuropathy in rats. Brain Research 2000;887:239–249.

[11] Bajaj S, Whiteman A, Brandner B. Transdermal drug delivery in pain management. Contin Educ Anaesth Crit Care Pain 2011;11:39–43.

[12] Barletta M, Ostenkamp SM, Taylor AC, Quandt J, Lascelles BDX, Messenger KM. The pharmacokinetics and analgesic effects of extended-release buprenorphine administered subcutaneously in healthy dogs. Journal of Veterinary Pharmacology and Therapeutics 2018;41:502–512.

[13] Benita S. Microencapsulation: Methods and Industrial Applications, Second Edition. CRC Press, 2005 p.

[14] Benvenutti DF, Bresolin TMB, Corrêa R, Giovagnoli S, Vivani R, Ricci M. A Novel Stabilizing Approach to Improve the Manufacturing of Biodegradable Microparticles Entrapping Plasticizing Active Molecules: the Case of 4-Methoxychalcone. J Pharm Innov 2019;14:159–175.

[15] Blasi P. Poly(lactic acid)/poly(lactic-co-glycolic acid)-based microparticles: an overview. J Pharm Investig 2019;49:337–346.

[16] Blasi P, D'Souza SS, Selmin F, DeLuca PP. Plasticizing effect of water on poly(lactide-co-glycolide). Journal of Controlled Release 2005;108:1–9.

[17] Boas RA, Villiger JW. CLINICAL ACTIONS OF FENTANYL AND BUPRENORPHINE: The Significance of Receptor Binding. British Journal of Anaesthesia 1985;57:192–196.

[18] Brodbelt DC, Taylor PM, Stanway GW. A comparison of preoperative morphine and buprenorphine for postoperative analgesia for arthrotomy in dogs. Journal of Veterinary Pharmacology and Therapeutics 1997;20:284–289.

[19] Budd K, Collett BJ. III. Old dog—new (ma)trix†. Br J Anaesth 2003;90:722–724.

[20] Bullingham RES, McQuay HJ, Moore A, Bennett MRD. Buprenorphine kinetics. Clinical Pharmacology & Therapeutics 1980;28:667–672.

[21] Busatto C, Pesoa J, Helbling I, Luna J, Estenoz D. Effect of particle size, polydispersity and polymer degradation on progesterone release from PLGA microparticles: Experimental and mathematical modeling. International Journal of Pharmaceutics 2018;536:360–369.

[22] BYDUREON® (exenatide extended-release) for injectable suspension. n.d.:49. https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/022200s026lbl.pdf. Accessed 2 Jul 2020.

[23] Çalış S, Bozdağ S, Kaş HS, Tunçay M, Hıncal AA. Influence of irradiation sterilization on poly(lactide-co-glycolide) microspheres containing anti-inflammatory drugs. Il Farmaco 2002;57:55–62.

[24] Carbone ET, Lindstrom KE, Diep S, Carbone L. Duration of Action of Sustained-Release Buprenorphine in 2 Strains of Mice. Journal of the American Association for Laboratory Animal Science 2012;51:815–819.

[25] Cary CD, Lukovsky-Akhsanov NL, Gallardo-Romero NF, Tansey CM, Ostergaard SD, Taylor Jr WD, Morgan CN, Powell N, Lathrop GW, Hutson CL. Pharmacokinetic Profiles of Meloxicam and Sustained-release Buprenorphine in Prairie Dogs (Cynomys ludovicianus). Journal of the American Association for Laboratory Animal Science 2017;56:160–165.

[26] Catbagan DL, Quimby JM, Mama KR, Rychel JK, Mich PM. Comparison of the efficacy and adverse effects of sustained-release buprenorphine hydrochloride following subcutaneous administration and buprenorphine hydrochloride following oral transmucosal administration in cats undergoing ovariohysterectomy. Am J Vet Res 2011;72:461–466.

[27] Chen Q, Shang Y, Xu Y, Li P, Li P, Liu G-L. Analgesic effect and pharmacological mechanism of fentanyl and butorphanol in a rat model of incisional pain. Journal of Clinical Anesthesia 2016;28:67–73.

[28] Cho C, Michailidis V, Lecker I, Collymore C, Hanwell D, Loka M, Danesh M, Pham C, Urban P, Bonin RP, Martin LJ. Evaluating analgesic efficacy and administration route following craniotomy in mice using the grimace scale. Sci Rep 2019;9. doi:10.1038/s41598-018-36897-w.

[29] Chum HH, Jampachairsri K, McKeon GP, Yeomans DC, Pacharinsak C, Felt SA. Antinociceptive Effects of Sustained-Release Buprenorphine in a Model of Incisional Pain in Rats (Rattus norvegicus). J Am Assoc Lab Anim Sci 2014;53:193–197.

[30] Clark TS, Clark DD, Jr RFH. Pharmacokinetic Comparison of Sustained-Release and Standard Buprenorphine in Mice. J Am Assoc Lab Anim Sci 2014;53:387–391.

[31] Coe MA, Lofwall MR, Walsh SL. Buprenorphine Pharmacology Review: Update on Transmucosal and Long-acting Formulations. Journal of Addiction Medicine 2019;13:93–103.

[32] Cone EJ, Gorodetzky CW, Yousefnejad D, Buchwald WF, Johnson RE. The metabolism and excretion of buprenorphine in humans. Drug Metab Dispos 1984;12:577–581.

[33] Cooper DM, Delong D, Gilleti CS. Analgesic Efficacy of Acetaminophen and Buprenorphine Administered in the Drinking Water of Rats. Journal of the American Association for Laboratory Animal Science 1997;36:58–62.

[34] Cowan A, Lewis JW, Macfarlane IR. Agonist and Antagonist Properties of Buprenorphine, a New Antinociceptive Agent. British Journal of Pharmacology n.d.;60:537–545.

[35] Cunningham C, Deacon R, Wells H, Boche D, Waters S, Diniz CP, Scott H, Rawlins JNP, Perry VH. Synaptic changes characterize early behavioural signs in the ME7 model of murine prion disease. European Journal of Neuroscience 2003;17:2147–2155.

[36] Dahan A, Yassen A, Bijl H, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M. Comparison of the respiratory effects of intravenous buprenorphine and fentanyl in humans and rats. Br J Anaesth 2005;94:825–834.

[37] Dahan A, Yassen A, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M. Buprenorphine induces ceiling in respiratory depression but not in analgesia. Br J Anaesth 2006;96:627–632.

[38] Davis MP. Buprenorphine in cancer pain. Support Care Cancer 2005;13:878–887.

[39] Deacon RM. Assessing nest building in mice. Nature Protocols 2006;1:1117–1119.

[40] Deacon RMJ. Housing, husbandry and handling of rodents for behavioral experiments. Nat Protoc 2006;1:936–946.

[41] Deacon RMJ, Croucher A, Rawlins JNP. Hippocampal cytotoxic lesion effects on species-typical behaviours in mice. Behavioural Brain Research 2002;132:203–213.

[42] Deacon RMJ, Penny C, Rawlins JNP. Effects of medial prefrontal cortex cytotoxic lesions in mice. Behavioural Brain Research 2003;139:139–155.

[43] Decapeptyl® SR 3 mgpowder for suspension for injection. n.d. Available: https://www.medicines.org.uk/emc/files/pil.963.pdf. Accessed 2 Jul 2020.

[44] Decapeptyl SR 3mg - Summary of Product Characteristics (SmPC) - (emc). n.d. Available: https://www.medicines.org.uk/EMC/medicine/868/SPC/Decapeptyl+SR+3mg. Accessed 2 Jul 2020.

[45] Drug Approval Package: SUBLOCADE (buprenorphine). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/209819Orig1s000TOC.cfm. Accessed 8 Jun 2020.

[46] D'Souza S, Faraj JA, Dorati R, DeLuca PP. Enhanced Degradation of Lactide-co-Glycolide Polymer with Basic Nucleophilic Drugs. Advances in Pharmaceutics 2015. doi:https://doi.org/10.1155/2015/154239.

[47] Elmowafy EM, Tiboni M, Soliman ME. Biocompatibility, biodegradation and biomedical applications of poly(lactic acid)/poly(lactic-co-glycolic acid) micro and nanoparticles. J Pharm Investig 2019;49:347–380.

[48] Enantone® Monats-Depot. n.d. Available: https://www.takeda.com/siteassets/de-de/home/what-we-do/packungsbeilagen/beilagen-2/gi_enatone_april2017.pdf. Accessed 2 Jul 2020.

[49] Erden N, Celebi N. Factors influencing release of salbutamol sulphate from poly(lactide-co-glycolide) microspheres prepared by water-in-oil-in-water emulsion technique. International Journal of Pharmaceutics 1996;137:57–66.

[50] Esposito E, Cortesi R, Bortolotti F, Menegatti E, Nastruzzi C. Production and characterization of biodegradable microparticles for the controlled delivery of proteinase inhibitors. International Journal of Pharmaceutics 1996;129:263–273.

[51] European Pharmacopoeia, 5.1.1. Methods of preparation of sterile products, in European Pharmacopoeia 8.0th ed. (2014), European directorate for the quality of medicines & health care.

[52] European Pharmacopoeia, 5.1.10. Guidelines for using the test of bacterial endotoxins, in European Pharmacopoeia 8.0th ed. (2014), European directorate for the quality of medicines & health care.

[53] European Pharmacopoeia, 5.4. Residual solvents, in European Pharmacopoeia 8.0th ed. (2014), European directorate for the quality of medicines & health care.

[54] Evangelista-Vaz R, Bergadano A, Arras M, Jirkof PD. Analgesic Efficacy of Subcutaneous–Oral Dosage of Tramadol after Surgery in C57BL/6J Mice. J Am Assoc Lab Anim Sci 2018;57:368–375.

[55] Evans HC, Easthope SE. Transdermal Buprenorphine: Drugs 2003;63:1999–2010.

[56] Fahmy TM, Fong PM, Goyal A, Saltzman WM. Targeted for drug delivery. Materials Today 2005;8:18–26.

[57] Filali M, Lalonde R. Age-related cognitive decline and nesting behavior in an APPswe/PS1 bigenic model of Alzheimer's disease. Brain Research 2009;1292:93–99.

[58] Flecknell PA, Roughan JV, Stewart R. Use of oral buprenorphine ('buprenorphine jello') for postoperative analgesia in rats-a clinical trial. Lab Anim 1999;33:169–174.

[59] Foley PL, Kendall LV, Turner PV. Clinical Management of Pain in Rodents. Comparative Medicine 2019;69:468–489.

[60] Foley PL, Liang H, Crichlow AR. Evaluation of a Sustained-Release Formulation of Buprenorphine for Analgesia in Rats. J Am Assoc Lab Anim Sci 2011;50:198–204.

[61] Fouad EA, EL-Badry M, Mahrous GM, Alanazi FK, Neau SH, Alsarra IA. The use of spray-drying to enhance celecoxib solubility. Drug Development and Industrial Pharmacy 2011;37:1463–1472.

[62] Franklin KBJ, Abbott FV. Techniques for Assessing the Effects of Drugs on Nociceptive Responses. In: Boulton AA, Baker GB, Greenshaw AJ, editors. Psychopharmacology. Neuromethods. Totowa, NJ: Humana Press, 1989. pp. 145–216. doi:10.1385/0-89603-129-2:145.

[63] Fu K, Pack DW, Klibanov AM, Langer R. Visual Evidence of Acidic Environment Within Degrading Poly(lactic-co-glycolic acid) (PLGA) Microspheres. Pharm Res. 2000 Jan 1;17(1):100-6

[64] Gades NM, Danneman PJ, Wixson SK, Tolley EA. The Magnitude and Duration of the Analgesic Effect of Morphine, Butorphanol, and Buprenorphine in Rats and Mice. Journal of the American Association for Laboratory Animal Science 2000;39:8–13.

[65] Gillingham MB, Clark MD, Dahly EM, Krugner-Higby LA, Ney DM. A Comparison of Two Opioid Analgesics for Relief of Visceral Pain Induced by Intestinal Resection in Rats. Journal of the American Association for Laboratory Animal Science 2001;40:21–26.

[66] Girón R, Abalo R, Goicoechea C, Martí n MI, Callado LF, Cano C, Goya P, Jagerovic N. Synthesis and opioid activity of new fentanyl analogs. Life Sciences 2002;71:1023–1034.

[67] GmbH RLS. Transtec PRO 70 Mikrogramm/h Transdermales Pflaster - PatientenInfo-Service. n.d. Available: https://www.patienteninfo-service.de/a-z-liste/t/transtec-pro-70-mikrogrammh-transdermales-pflaster/. Accessed 24 Jun 2020.

[68] Goldkuhl R, Carlsson H-E, Hau J, Abelson KSP. Effect of Subcutaneous Injection and Oral Voluntary Ingestion of Buprenorphine on Post-Operative Serum Corticosterone Levels in Male Rats. Eur Surg Res 2008;41:272–278.

[69] Goldkuhl R, Jacobsen KR, Kalliokoski O, Hau J, Abelson KSP. Plasma concentrations of corticosterone and buprenorphine in rats subjected to jugular vein catheterization. Lab Anim 2010;44:337–343.

[70] Guarnieri M, Brayton C, Sarabia-Estrada R, Tyler B, McKnight P, DeTolla L. Subcutaneous Implants of a Cholesterol-Triglyceride-Buprenorphine Suspension in Rats. J Vet Med 2017;2017. doi:10.1155/2017/3102567.

[71] Guarnieri M, Brayton C, Tyler BM. A Long-Term Study of a Lipid-Buprenorphine Implant in Rats. J Vet Med 2018;2018. doi:10.1155/2018/2616152.

[72] Guarnieri M, Tyler BM, DeTolla L, Zhao M, Kobrin B. Subcutaneous implants for long-acting drug therapy in laboratory animals may generate unintended drug reservoirs. J Pharm Bioallied Sci 2014;6:38–42.

[73] Guzman DS-M, Knych HK, Olsen GH, Paul-Murphy JR. Pharmacokinetics of a Sustained Release Formulation of Buprenorphine After Intramuscular and Subcutaneous Administration to American Kestrels (Falco sparverius). J Avian Med Surg 2017;31:102–107.

[74] Hagen NA, Biondo P, Stiles C. Assessment and management of breakthrough pain in cancer patients: Current approaches and emerging research. Current Science Inc 2008;12:241–248.

[75] Han E-J, Chung A-H, Oh I-J. Analysis of residual solvents in poly(lactide-co-glycolide) nanoparticles. Journal of Pharmaceutical Investigation 2012;42:251–256.

[76] Hausberger AG, Kenley RA, DeLuca PP. Gamma Irradiation Effects on Molecular Weight and in Vitro Degradation of Poly(D,L-Lactide-CO-Glycolide) Microparticles. Pharm Res 1995;12:851–856.

[77] Hayes AG, Skingle M, Tyers MB. Reversal by beta-funaltrexamine of the antinociceptive effect of opioid agonists in the rat. Br J Pharmacol 1986;88:867–872.

[78] Healy JR, Tonkin JL, Kamarec SR, Saludes MA, Ibrahim SY, Matsumoto RR, Wimsatt JH. Evaluation of an improved sustained-release buprenorphine formulation for use in mice. American Journal of Veterinary Research 2014;75:619–625.

[79] Herndon NL, Bandyopadhyay S, Hod EA, Prestia KA. Sustained-Release Buprenorphine Improves Postsurgical Clinical Condition but Does Not Alter Survival or Cytokine Levels in a Murine Model of Polymicrobial Sepsis. Comp Med 2016;66:455–462.

[80] Hestehave S, Munro G, Pedersen TB, Abelson KSP. Antinociceptive effects of voluntarily ingested buprenorphine in the hot-plate test in laboratory rats. Lab Anim 2017;51:264–272.

[81] Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C. Severity classification of repeated isoflurane anesthesia in C57BL/6JRj mice—Assessing the degree of distress. PLOS ONE 2017;12:e0179588.

[82] Hovard AMB, Teilmann AC, Hau J, Abelson KSP. The applicability of a gel delivery system for self-administration of buprenorphine to laboratory mice: Laboratory Animals 2014. doi:10.1177/0023677214551108.

[83] Huang P, Kehner GB, Cowan A, Liu-Chen L-Y. Comparison of Pharmacological Activities of Buprenorphine and Norbuprenorphine: Norbuprenorphine Is a Potent Opioid Agonist. J Pharmcol Exp Ther. 2001 May 1;297(2):688-95.

[84] Huang X, Brazel CS. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. Journal of Controlled Release 2001;73:121–136.

[85] Ide S, Minami M, Satoh M, Uhl GR, Sora I, Ikeda K. Buprenorphine Antinociception is Abolished, but Naloxone-Sensitive Reward is Retained, in μ -Opioid Receptor Knockout Mice. Neuropsychopharmacology 2004;29:1656–1663.

[86] Iribarne C, Picart D, Dréano Y, Bail J-P, Berthou F. Involvement of cytochrome P450 3A4 in N-dealkylation of buprenorphine in human liver microsomes. Life Sciences 1997;60:1953–1964.

[87] Jablonski P, Howden BO, Baxter K. Influence of buprenorphine analgesia on post-operative recovery in two strains of rats. Lab Anim 2001;35:213–222.

[88] Jagerovic N, Cano C, Elguero J, Goya P, Callado LF, Javier Meana J, Girón R, Abalo R, Ruiz D, Goicoechea C, Isabel Martín M[a]. Long-Acting Fentanyl Analogues: Synthesis and Pharmacology of N-(1-Phenylpyrazolyl)-N-(1-phenylalkyl-4-piperidyl)propanamides. Bioorganic & Medicinal Chemistry 2002;10:817–827.

[89] Jain A, Kunduru KR, Basu A, Mizrahi B, Domb AJ, Khan W. Injectable formulations of poly(lactic acid) and its copolymers in clinical use. Advanced Drug Delivery Reviews 2016;107:213–227.

[90] Jain R, Shah NH, Malick AW, Rhodes CT. Controlled Drug Delivery by Biodegradable Poly(Ester) Devices: Different Preparative Approaches. Drug Development and Industrial Pharmacy 1998;24:703–727.

[91] Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 2000;21:2475–2490.

[92] Jalil R, Nixon JR. Microencapsulation using poly(DL-lactic acid) III: Effect of polymer molecular weight on the release kinetics. Journal of Microencapsulation 1990;7:357–374.

[93] Jaraswekin S, Prakongpan S, Bodmeier R. Effect of poly(lactide-co-glycolide) molecular weight on the release of dexamethasone sodium phosphate from microparticles. Journal of Microencapsulation 2007;24:117–128.

[94] Jasinski DR, Pevnick JS, Griffith JD. Human Pharmacology and Abuse Potential of the Analgesic Buprenorphine: A Potential Agent for Treating Narcotic Addiction. Arch Gen Psychiatry 1978;35:501–516.

[95] Jensen DMK, Cun D, Maltesen MJ, Frokjaer S, Nielsen HM, Foged C. Spray drying of siRNA-containing PLGA nanoparticles intended for inhalation. Journal of Controlled Release 2010;142:138–145.

[96] Jessen L, Christensen S, Bjerrum OJ. The antinociceptive efficacy of buprenorphine administered through the drinking water of rats. Lab Anim 2007;41:185–196.

[97] Jirkof P. Burrowing and nest building behavior as indicators of well-being in mice. J Neurosci Methods 2014;234:139–46.

[98] Jirkof P, Cesarovic N, Rettich A, Nicholls F, Seifert B, Arras M. Burrowing behavior as an indicator of post-laparotomy pain in mice. Front Behav Neurosci 2010;4:165.

[99] Jirkof P, Fleischmann T, Cesarovic N, Rettich A, Vogel J, Arras M. Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring. Laboratory Animals 2013;47:153–161.

[100] Jirkof P, Leucht K, Cesarovic N, Caj M, Nicholls F, Rogler G, Arras M, Hausmann M. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. Lab Anim 2013;47:274–83.

[101] Johnson RA. Voluntary Running-Wheel Activity, Arterial Blood Gases, and Thermal Antinociception in Rats after 3 Buprenorphine Formulations. Journal of the American Association for Laboratory Animal Science 2016;55:306–311.

[102] Johnson RE, Chutuape MA, Strain EC, Walsh SL, Stitzer ML, Bigelow GE. A Comparison of Levomethadyl Acetate, Buprenorphine, and Methadone for Opioid Dependence. New England Journal of Medicine 2000;343:1290–1297.

[103] Johnson RE, Eissenberg T, Stitzer ML, Strain EC, Liebson IA, Bigelow GE. A placebo controlled clinical trial of buprenorphine as a treatment for opioid dependence. Drug and Alcohol Dependence 1995;40:17–25.

[104] Kalliokoski O, Jacobsen KR, Hau J, Abelson KSP. Serum concentrations of buprenorphine after oral and parenteral administration in male mice. The Veterinary Journal 2011;187:251–254.

[105] Kamei J, Ohsawa M, Hayashi S-S, Nakanishi Y. Effect of chronic pain on morphine-induced respiratory depression in mice. Neuroscience 2011;174:224–233.

[106] Kamei J, Saitoh A, Suzuki T, Misawa M, Nagase H, Kasuya Y. Buprenorphine exerts its antinocicepttve activity via μ1-opioid receptors. Life Sciences 1995;56:PL285–PL290.

[107] Karlsson OJ, Stubbs JM, Karlsson LE, Sundberg DC. Estimating diffusion coefficients for small molecules in polymers and polymer solutions. Polymer 2001;42:4915–4923.

[108] Kendall LV, Hansen RJ, Dorsey K, Kang S, Lunghofer PJ, Gustafson DL. Pharmacokinetics of Sustained-Release Analgesics in Mice. J Am Assoc Lab Anim Sci 2014;53:478–484.

[109] Kendall LV, Wegenast DJ, Smith BJ, Dorsey KM, Kang S, Lee NY, Hess AM. Efficacy of Sustained-Release Buprenorphine in an Experimental Laparotomy Model in Female Mice. J Am Assoc Lab Anim Sci 2016;55:66–73.

[110] Kim HK, Park TG. Microencapsulation of human growth hormone within biodegradable polyester microspheres: Protein aggregation stability and incomplete release mechanism. Biotechnology and Bioengineering 1999;65:659–667.

[111] Klune CB, Larkin AE, Leung VSY, Pang D. Comparing the Rat Grimace Scale and a composite behaviour score in rats. PLOS ONE 2019;14:e0209467.

[112] Koltzenburg M, Pokorny R, Gasser UE, Richarz U. Differential sensitivity of three experimental pain models in detecting the analgesic effects of transdermal fentanyl and buprenorphine. Pain 2006;126:165–174.

[113] Kongara K, Chambers JP, Johnson CB. Effects of tramadol, morphine or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. New Zealand Veterinary Journal 2012;60:129–135.

[114] Kumar MNVR. Handbook of Polyester Drug Delivery Systems. CRC Press, 2017 p.

[115] Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, LaCroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, Maagdenberg AMJM van den, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nature Methods 2010;7:447–449.

[116] Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev 2001;53:597–652.

[117] Leach MC, Forrester AR, Flecknell PA. Influence of preferred foodstuffs on the antinociceptive effects of orally administered buprenorphine in laboratory rats. Lab Anim 2010;44:54–58.

[118] Leon RAL, Somasundar A, Badruddoza AZM, Khan SA. Microfluidic Fabrication of Multi-Drug-Loaded Polymeric Microparticles for Topical Glaucoma Therapy. Particle & Particle Systems Characterization 2015;32:567–572.

[119] Lijam N, Paylor R, McDonald MP, Crawley JN, Deng C-X, Herrup K, Stevens KE, Maccaferri G, McBain CJ, Sussman DJ, Wynshaw-Boris A. Social Interaction and Sensorimotor Gating Abnormalities in Mice Lacking Dvl1. Cell 1997;90:895–905.

[120] Likar R. Transdermal buprenorphine in the management of persistent pain – safety aspects. Ther Clin Risk Manag 2006;2:115–125.

[121] Liles JH, Flecknell PA. The effects of buprenorphine, nalbuphine and butorphanol alone or following halothane anaesthesia on food and water consumption and locomotor movement in rats. Lab Anim 1992;26:180–189.

[122] Liles JH, Flecknell PA. The influence of buprenorphine or bupivacaine on the post-operative effects of laparotomy and bile-duct ligation in rats. Lab Anim 1993;27:374–380.

[123] Liles JH, Flecknell PA, Roughan J, Cruz-Madorran I. Influence of oral buprenorphine, oral naltrexone or morphine on the effects of laparotomy in the rat. Lab Anim 1998;32:149–161.

[124] Ling W, Casadonte P, Bigelow G, Kampman KM, Patkar A, Bailey GL, Rosenthal RN, Beebe KL. Buprenorphine Implants for Treatment of Opioid Dependence: A Randomized Controlled Trial. JAMA 2010;304:1576–1583.

[125] Liu R, Huang S-S, Wan Y-H, Ma G-H, Su Z-G. Preparation of insulin-loaded PLA/PLGA microcapsules by a novel membrane emulsification method and its release in vitro. Colloids and Surfaces B: Biointerfaces 2006;51:30–38.

[126] Liu R, Ma G, Meng F-T, Su Z-G. Preparation of uniform-sized PLA microcapsules by combining Shirasu Porous Glass membrane emulsification technique and multiple emulsion-solvent evaporation method. Journal of Controlled Release 2005;103:31–43.

[127] Liu Y, Hu C-Q. Establishment of a knowledge base for identification of residual solvents in pharmaceuticals. Analytica Chimica Acta 2006;575:246–254.

[128] Loo SCJ, Ooi CP, Boey YCF. Radiation effects on poly(lactide-co-glycolide) (PLGA) and poly(l-lactide) (PLLA). Polymer Degradation and Stability 2004;83:259–265.

[129] Luan X, Bodmeier R. Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems. Journal of Controlled Release 2006;110:266–272.

[130] LUPRON DEPOT (leuprolide acetate for depot suspension). n.d.:31. https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/020517s036_019732s041lbl.pdf. Accessed 2 Jul 2020.

[131] Ma G, Nagai M, Omi S. Preparation of uniform poly(lactide) microspheres by employing the Shirasu Porous Glass (SPG) emulsification technique. Colloids and Surfaces A: Physicochemical and Engineering Aspects 1999;153:383–394.

[132] Makadia HK, Siegel SJ. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. Polymers 2011;3:1377–1397.

[133] Maltesen MJ, Bjerregaard S, Hovgaard L, Havelund S, van de Weert M. Quality by design – Spray drying of insulin intended for inhalation. European Journal of Pharmaceutics and Biopharmaceutics 2008;70:828–838.

[134] Mandal TK, Tenjarla S. Preparation of biodegradable microcapsules of zidovudine using solvent evaporation: Effect of the modification of aqueous phase. International Journal of Pharmaceutics 1996;137:187–197.

[135] Martin LBE, Thompson AC, Martin T, Kristal MB. Analgesic Efficacy of Orally Administered Buprenorphine in Rats. Comparative Medicine 2001;51:43–48.

[136] Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther 1976;197:517–532.

[137] Mastrocinque S, Fantoni DT. A comparison of preoperative tramadol and morphine for the control of early postoperative pain in canine ovariohysterectomy. Veterinary Anaesthesia and Analgesia 2003;30:220–228.

[138] Matsumiya LC, Sorge RE, Sotocinal SG, Tabaka JM, Wieskopf JS, Zaloum A, King OD, Mogil JS. Using the Mouse Grimace Scale to Reevaluate the Efficacy of Postoperative Analgesics in Laboratory Mice. J Am Assoc Lab Anim Sci 2012;51:42–49.

[139] Medicine C for V. The Index of Legally Marketed Unapproved New Animal Drugs for Minor Species. FDA 2020. Available: https://www.fda.gov/animal-veterinary/minor-useminor-species/index-legally-marketed-unapproved-new-animal-drugs-minor-species. Accessed 13 Jun 2020.

[140] Meert TF, Vermeirsch HA. A preclinical comparison between different opioids: antinociceptive versus adverse effects. Pharmacology Biochemistry and Behavior 2005;80:309–326.

[141] Meijer MK, Spruijt BM, Zutphen LFM van, Baumans V. Effect of restraint and injection methods on heart rate and body temperature in mice: Laboratory Animals 2016. doi:10.1258/002367706778476370.

[142] MGS Manual.pdf. n.d. Available: https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/MGS%20Manual.pdf. Accessed 1 Jul 2020.

[143] Middleton JC, Tipton AJ. Synthetic biodegradable polymers as orthopedic devices. Biomaterials 2000;21:2335–2346.

[144] Miller A, Kitson G, Skalkoyannis B, Leach M. The effect of isoflurane anaesthesia and buprenorphine on the mouse grimace scale and behaviour in CBA and DBA/2 mice. Appl Anim Behav Sci 2015;172:58–62.

[145] Miller AL, Golledge HDR, Leach MC. The Influence of Isoflurane Anaesthesia on the Rat Grimace Scale. PLOS ONE 2016;11:e0166652.

[146] Miller AL, Kitson GL, Skalkoyannis B, Flecknell PA, Leach MC. Using the mouse grimace scale and behaviour to assess pain in CBA mice following vasectomy. Applied Animal Behaviour Science 2016;181:160–165.

[147] Miller AL, Leach MC. The effect of handling method on the mouse grimace scale in two strains of laboratory mice. Lab Anim 2016;50:305–307.

[148] Miller AL, Leach MC. The Mouse Grimace Scale: A Clinically Useful Tool? PLOS ONE 2015;10:e0136000.

[149] Miller RA, Brady JM, Cutright DE. Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. Journal of Biomedical Materials Research 1977;11:711–719.

[150] Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV. Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. Journal of Controlled Release 2007;119:77–85.

[151] Mogil JS. Interaction between sex and genotype in the mediation of pain and pain inhibition. Seminars in Pain Medicine 2003;1:197–205.

[152] Mogil JS. The genetic mediation of individual differences in sensitivity to pain and its inhibition. Proceedings of the National Academy of Sciences 1999;96:7744–7751.

[153] Mogil JS, Ritchie J, Sotocinal SG, Smith SB, Croteau S, Levitin DJ, Naumova AK. Screening for pain phenotypes: Analysis of three congenic mouse strains on a battery of nine nociceptive assays. Pain 2006;126:24–34.

[154] Mogil JS, Wilson SG, Bon K, Eun Lee S, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Mo Chung J, Devor M. Heritability of nociception I: Responses of 11 inbred mouse strains on 12 measures of nociception. Pain 1999;80:67–82.

[155] Molina-Cimadevila MJ, Segura S, Merino C, Ruiz-Reig N, Andrés B, de Madaria E. Oral self-administration of buprenorphine in the diet for analgesia in mice. Lab Anim 2014;48:216–224.

[156] Moll X, Fresno L, García F, Prandi D, Andaluz A. Comparison of subcutaneous and transdermal administration of buprenorphine for pre-emptive analgesia in dogs undergoing elective ovariohysterectomy. The Veterinary Journal 2011;187:124–128.

[157] Molter CM, Barbosa L, Johnson S, Knych HK, Chinnadurai SK, Wack RF. Pharmacokinetics of a single subcutaneous dose of sustained release buprenorphine in northern elephant seals (Mirounga angustirostris). J Zoo Wildl Med 2015;46:52–61.

[158] Murrell JC, Robertson SA, Taylor PM, McCown JL, Bloomfield M, Sear JW. Use of a transdermal matrix patch of buprenorphine in cats: preliminary pharmacokinetic and pharmacodynamic data. Veterinary Record 2007;160:578–583.

[159] Nasser AF, Greenwald MK, Vince B, Fudala PJ, Twumasi-Ankrah P, Liu Y, Jones J, Heidbreder C. Sustained-Release Buprenorphine (RBP-6000) Blocks the Effects of Opioid Challenge With Hydromorphone in Subjects With Opioid Use Disorder. J Clin Psychopharmacol 2016;36:18–26.

[160] Negus SS, Bidlack JM, Mello NK, Furness MS, Rice KC, Brandt MR. Delta opioid antagonist effects of buprenorphine in rhesus monkeys. Behavioural Pharmacology 2002;13:557–570.

[161] Nihant N, Grandfils C, Jérôme R, Teyssié P. Microencapsulation by coacervation of poly(lactide-co-glycolide) IV. Effect of the processing parameters on coacervation and encapsulation. Journal of Controlled Release 1995;35:117–125.

[162] Nunamaker EA, Goldman JL, Adams CR, Fortman JD. Evaluation of Analgesic Efficacy of Meloxicam and 2 Formulations of Buprenorphine after Laparotomy in Female Sprague–Dawley Rats. J Am Assoc Lab Anim Sci 2018;57:498–507.

[163] Nunamaker EA, Halliday LC, Moody DE, Fang WB, Lindeblad M, Fortman JD. Pharmacokinetics of 2 Formulations of Buprenorphine in Macaques (Macaca mulatta and Macaca fascicularis). Journal of the American Association for Laboratory Animal Science 2013;52:48–56.

[164] Nunamaker EA, Stolarik DF, Ma J, Wilsey AS, Jenkins GJ, Medina CL. Clinical Efficacy of Sustained-Release Buprenorphine with Meloxicam for Postoperative Analgesia in Beagle Dogs Undergoing Ovariohysterectomy. J Am Assoc Lab Anim Sci 2014;53:494–501.

[165] Nutropin Depot- [somatropin (rDNA origin) for injectable suspension]. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/21075s008lbl.pdf. Accessed 2 Jul 2020.

[166] Ohtani M, Kotaki H, Nishitateno K, Sawada Y, Iga T. Kinetics of Respiratory Depression in Rats Induced by Buprenorphine and its Metabolite, Norbuprenorphine. J Pharmacol Exp Ther 1997;281:428–433.

[167] Ohtani M, Kotaki H, Sawada Y, Iga T. Comparative analysis of buprenorphine- and norbuprenorphine-induced analgesic effects based on pharmacokinetic-pharmacodynamic modeling. J Pharmacol Exp Ther 1995;272:505–510.

[168] Ohtani M, Kotaki H, Uchino K, Sawada Y, Iga T. Pharmacokinetic analysis of enterohepatic circulation of buprenorphine and its active metabolite, norbuprenorphine, in rats. Drug Metab Dispos 1994;22:2–7.

[169] Oliver VL, Athavale S, Simon KE, Kendall LV, Nemzek JA, Lofgren JL. Evaluation of Pain Assessment Techniques and Analgesia Efficacy in a Female Guinea Pig (Cavia porcellus) Model of Surgical Pain. Journal of the American Association for Laboratory Animal Science 2017;56:425–435.

[170] Otto KA, Steiner KHS, Zailskas F, Wippermann B. Comparison of the postoperative analgesic effects of buprenorphine and piritramide following experimental orthopaedic surgery in sheep. Journal of Experimental Animal Science 2000;41:133–143.

[171] Page CD, Sarabia-Estrada R, Hoffman RJ, Lo C-P, Gades NM. Lack of Absorption of a Sustained-release Buprenorphine Formulation Administered Subcutaneously to Athymic Nude Rats. 2019. doi:info:doi/10.30802/AALAS-JAALAS-19-000013.

[172] Pai Kasturi S, Qin H, Thomson KS, El-Bereir S, Cha S, Neelapu S, Kwak LW, Roy K. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. Journal of Controlled Release 2006;113:261–270.

[173] Park K, Skidmore S, Hadar J, Garner J, Park H, Otte A, Soh BK, Yoon G, Yu D, Yun Y, Lee BK, Jiang X, Wang Y. Injectable, long-acting PLGA formulations: Analyzing PLGA and understanding microparticle formation. Journal of Controlled Release 2019;304:125–134.

[174] Park TG. Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition. Biomaterials 1995;16:1123–1130.

[175] Pathan H, Williams J. Basic opioid pharmacology: an update. Br J Pain 2012;6:11–16.

[176] Paudel A, Worku ZA, Meeus J, Guns S, Van den Mooter G. Manufacturing of solid dispersions of poorly water soluble drugs by spray drying: Formulation and process considerations. International Journal of Pharmaceutics 2013;453:253–284.

[177] Periti P, Mazzei T, Mini E. Clinical Pharmacokinetics of Depot Leuprorelin. Clin Pharmacokinet 2002;41:485–504.

[178] Piacentini E, Drioli E, Giorno L. Membrane emulsification technology: Twenty-five years of inventions and research through patent survey. Journal of Membrane Science 2014;468:410–422.

[179] Pieper K, Schuster T, Levionnois O, Matis U, Bergadano A. Antinociceptive efficacy and plasma concentrations of transdermal buprenorphine in dogs. The Veterinary Journal 2011;187:335–341.

[180] R NG, L D, C S, N V, M NB, V L. Lyophilization/Freeze Drying - An Review. International Journal of Novel Trends in Pharmaceutical Sciences 2013;3:87–98.

[181] Rathbone MJ, Hadgraft J, Roberts MS eds. Modified-release drug delivery technology. New York: Marcel Dekker, 2003 p.

[182] Rawat A, Stippler E, Shah VP, Burgess DJ. Validation of USP apparatus 4 method for microsphere in vitro release testing using Risperdal® Consta®. International Journal of Pharmaceutics 2011;420:198–205.

[183] Richards ML, Sadée W. Buprenorphine is an Antagonist at the $\varkappa$ Opioid Receptor. Pharm Res 1985;2:178–181.

[184] RISPERDAL® CONSTA® (risperidone) LONG-ACTING INJECTION. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021346_s31_s35_s38_s39lbl.pdf . Accessed 2 Jul 2020.

[185] RISPERDAL CONSTA® INTRAMUSCULAR INJECTION Consumer Medicine Information (CMI). n.d. Available: https://medicines.org.au/files/jccrisco.pdf. Accessed 2 Jul 2020.

[186] Rodriguez NA, Cooper DM, Risdahl JM. Antinociceptive Activity of and Clinical Experience with Buprenorphine in Swine. Journal of the American Association for Laboratory Animal Science 2001;40:17–20.

[187] Romberg R, Sarton E, Teppema L, Matthes HWD, Kieffer BL, Dahan A. Comparison of morphine-6-glucuronide and morphine on respiratory depressant and antinociceptive responses in wild type and μ-opioid receptor deficient mice. Br J Anaesth 2003;91:862–870.

[188] Rose JD, Woodbury CJ. Animal Models of Nociception and Pain. In: Conn PM, editor. Sourcebook of Models for Biomedical Research. Totowa, NJ: Humana Press, 2008. pp. 333–339. doi:10.1007/978-1-59745-285-4_36.

[189] Rosenthal RN, Ling W, Casadonte P, Vocci F, Bailey GL, Kampman K, Patkar A, Chavoustie S, Blasey C, Sigmon S, Beebe KL. Buprenorphine Implants for Treatment of Opioid Dependence: Randomized Comparison to Placebo and Sublingual Buprenorphine/Naloxone. Addiction 2013;108:2141–2149.

[190] Roughan JV, Bertrand HGMJ, Isles HM. Meloxicam prevents COX-2-mediated post-surgical inflammation but not pain following laparotomy in mice. European Journal of Pain 2016;20:231–240.

[191] Roughan JV, Flecknell PA. Behaviour-based assessment of the duration of laparotomy-induced abdominal pain and the analgesic effects of carprofen and buprenorphine in rats. Behavioural Pharmacology 2004;15:461–472.

[192] Roughan JV, Flecknell PA. Buprenorphine: a reappraisal of its antinociceptive effects and therapeutic use in alleviating post-operative pain in animals: Laboratory Animals 2016. doi:10.1258/002367702320162423.

[193] Ryder D. Eligard® (leuprolide acetate for injectable solution) 7.5 mg, 22.5 mg, 30 mg, and 45mg. n.d.:62. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/021343Orig1s033.pdf. Accessed 4 Jul 2020.

[194] Sadée W, Rosenbaum JS, Herz A. Buprenorphine: differential interaction with opiate receptor subtypes in vivo. J Pharmacol Exp Ther 1982;223:157–162.

[195] Salvacyl. n.d. Available: https://www.medicines.org.uk/emc/files/pil.5804.pdf. Accessed 4 Jul 2020.

[196] Sandostatin LAR®Depot(octreotide acetate for injectable suspension). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/21008scs010_sandostatin_lbl.pdf. Accessed 2 Jul 2020.

[197] Sauer M, Fleischmann T, Lipiski M, Arras M, Jirkof P. Buprenorphine via drinking water and combined oral-injection protocols for pain relief in mice. Applied Animal Behaviour Science 2016;185:103–112.

[198] Schildhaus N, Trink E, Polson C, DeTolla L, Tyler BM, Jallo GI, Tok S, Guarnieri M. Thermal latency studies in opiate-treated mice. J Pharm Bioallied Sci 2014;6:43–47.

[199] Schoubben A, Ricci M, Giovagnoli S. Meeting the unmet: from traditional to cutting-edge techniques for poly lactide and poly lactide-co-glycolide microparticle manufacturing. J Pharm Investig 2019;49:381–404.

[200] Selmin F, Blasi P, DeLuca PP. Accelerated Polymer Biodegradation of Risperidone Poly(d, l-Lactide-Co-Glycolide) Microspheres. AAPS PharmSciTech 2012;13:1465–1472.

[201] Seymour TL, Adams SC, Felt SA, Jampachaisri K, Yeomans DC, Pacharinsak C. Postoperative Analgesia Due to Sustained-Release Buprenorphine, Sustained-Release Meloxicam, and Carprofen Gel in a Model of Incisional Pain in Rats (Rattus norvegicus). J Am Assoc Lab Anim Sci 2016;55:300–305.

[202] SIGNIFOR® LAR (pasireotide). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/203255s000lbl.pdf. Accessed 4 Jul 2020.

[203] Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. Journal of Controlled Release 2003;90:261–280.

[204] Sittl R, Griessinger N, Likar R. Analgesic efficacy and tolerability of transdermal buprenorphine in patients with inadequately controlled chronic pain related to cancer and other disorders: A multicenter, randomized, double-blind, placebo-controlled trial. Clinical Therapeutics 2003;25:150–168.

[205] Smith BJ, Wegenast DJ, Hansen RJ, Hess AM, Kendall LV. Pharmacokinetics and Paw Withdrawal Pressure in Female Guinea Pigs (Cavia porcellus) Treated with Sustained-Release Buprenorphine and Buprenorphine Hydrochloride. J Am Assoc Lab Anim Sci 2016;55:789–793.

[206] Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin J-S, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS. Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male But Not Female Mice. J Neurosci 2011;31:15450–15454.

[207] Sorge RE, Mapplebeck JCS, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin J-S, Sotocinal SG, Chen D, Yang M, Shi XQ, Huang H, Pillon NJ, Bilan PJ, Tu Y, Klip A, Ji R-R, Zhang J, Salter MW, Mogil JS. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nature Neuroscience 2015;18:1081–1083.

[208] Spenlehauer G, Vert M, Benoit JP, Boddaert A. In vitro and In vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials 1989;10:557–563.

[209] Speth RC, Smith Smith MS, Brogan RS. Regarding the Inadvisability of Administering Postoperative Analgesics in the Drinking Water of Rats (Rattus norvegicus). Journal of the American Association for Laboratory Animal Science 2001;40:15–17.

[210] Stanway GW, Taylor PM, Brodbelt DC. A preliminary investigation comparing pre-operative morphine and buprenorphine for postoperative analgesia and sedation in cats. Veterinary Anaesthesia and Analgesia 2002;29:29–35.

[211] Stasiak KL, Maul D, French E, Hellyer PW, Vandewoude S. Species-Specific Assessment of Pain in Laboratory Animals. Journal of the American Association for Laboratory Animal Science 2003;42:13–20.

[212] Stokes EL, Flecknell PA, Richardson CA. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim 2009;43:149–154.

[213] SUBLOCADE (buprenorphine extended-release) injection, for subcutaneous use. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/209819s000lbl.pdf. Accessed 3 Jul 2020.

[214] suprefact-depot.pdf. n.d. Available: http://products.sanofi.ca/en/suprefact-depot.pdf. Accessed 2 Jul 2020.

[215] Taylor BF, Ramirez HE, Battles AH, Andrutis KA, Neubert JK. Analgesic Activity of Tramadol and Buprenorphine after Voluntary Ingestion by Rats (Rattus norvegicus). J Am Assoc Lab Anim Sci 2016;55:74–82.

[216] Thiede AJ, Garcia KD, Stolarik DF, Ma J, Jenkins GJ, Nunamaker EA. Pharmacokinetics of Sustained-Release and Transdermal Buprenorphine in Göttingen Minipigs (Sus scrofa domestica). Journal of the American Association for Laboratory Animal Science 2014;53:692–699.

[217] Thomasin C, Nam-Trân H, Merkle HP, Gander B. Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical considerations. Journal of Pharmaceutical Sciences 1998;87:259–268.

[218] Thompson AC, DiPirro JM, Sylvester AR, Martin LB, Kristal MB. Lack of Analgesic Efficacy in Female Rats of the Commonly Recommended Oral Dose of Buprenorphine. Journal of the American Association for Laboratory Animal Science 2006;45:13–16.

[219] Thompson AC, Kristal MB, Sallaj A, Acheson A, Martin LBE, Martin T. Analgesic Efficacy of Orally Administered Buprenorphine in Rats: Methodologic Considerations. Comparative Medicine 2004;54:293–300.

[220] Tinsley-Bown AM, Fretwell R, Dowsett AB, Davis SL, G.H. Farrar. Formulation of poly(d,l-lactic-co-glycolic acid) microparticles for rapid plasmid DNA delivery. Journal of Controlled Release 2000;66:229–241.

[221] Tracy MA, Ward KL, Firouzabadian L, Wang Y, Dong N, Qian R, Zhang Y. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in vitro. Biomaterials 1999;20:1057–1062.

[222] Traul KA, Romero JB, Brayton C, DeTolla L, Forbes-McBean N, Halquist MS, Karnes HT, Sarabia-Estrada R, Tomlinson MJ, Tyler BM, Ye X, Zadnik P, Guarnieri M. Safety studies of post-surgical buprenorphine therapy for mice. Lab Anim 2015;49:100–110.

[223] Traynor JR, Nahorski SR. Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol Pharmacol 1995;47:848–854.

[224] TRELSTAR® (triptorelin pamoate for injectable suspension). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020715s024,021288s021,022437s002lbl.pdf. Accessed 2 Jul 2020.

[225] TRIPTODUR (triptorelin). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/208956s000lbl.pdf. Accessed 4 Jul 2020.

[226] VIVITROL® (naltrexone for extended-release injectable suspension) Intramuscular. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021897s015lbl.pdf. Accessed 2 Jul 2020.

[227] Vladisavljević GT. Structured microparticles with tailored properties produced by membrane emulsification. Advances in Colloid and Interface Science 2015;225:53–87.

[228] Waite ME, Tomkovich A, Quinn TL, Schumann AP, Dewberry LS, Totsch SK, Sorge RE. Efficacy of Common Analgesics for Postsurgical Pain in Rats. Journal of the American Association for Laboratory Animal Science 2015;54:420–425.

[229] Walkowiak KJ, Graham ML. Pharmacokinetics and Antinociceptive Activity of Sustained-Release Buprenorphine in Sheep. J Am Assoc Lab Anim Sci 2015;54:763–768.

[230] Walsh SL, Preston KL, Stitzer ML, Cone EJ, Bigelow GE. Clinical pharmacology of buprenorphine: Ceiling effects at high doses. Clinical Pharmacology & Therapeutics 1994;55:569–580.

[231] Walter E, Dreher D, Kok M, Thiele L, Kiama SG, Gehr P, Merkle HP. Hydrophilic poly(dl-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. Journal of Controlled Release 2001;76:149–168.

[232] Wan F, Yang M. Design of PLGA-based depot delivery systems for biopharmaceuticals prepared by spray drying. International Journal of Pharmaceutics 2016;498:82–95.

[233] Wang J, Helder L, Shao J, Jansen JA, Yang M, Yang F. Encapsulation and release of doxycycline from electrospray-generated PLGA microspheres: Effect of polymer end groups. International Journal of Pharmaceutics 2019;564:1–9.

[234] Wang Y, Burgess DJ. Influence of storage temperature and moisture on the performance of microsphere/hydrogel composites. International Journal of Pharmaceutics 2013;454:310–315.

[235] Wang Y, Tran KK, Shen H, Grainger DW. Selective local delivery of RANK siRNA to bone phagocytes using bone augmentation biomaterials. Biomaterials 2012;33:8540–8547.

[236] Watson PJQ, McQUAY HJ, Bullingham RES, Allen MC, Moore RA. SINGLE-DOSE COMPARISON OF BUPRENORPHINE 0.3 AND 0.6 MG I.V. GIVEN AFTER OPERATION: CLINICAL EFFECTS AND PLASMA CONCENTRATIONS. British Journal of Anaesthesia 1982;54:37–43.

[237] van de Weert M, Hennink WE, Jiskoot W. Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles. Pharm Res. 2000 Oct 1;17(10):1159-67.

[238] Witschi C, Doelker E. Residual solvents in pharmaceutical products: acceptable limits, influences on physicochemical properties, analytical methods and documented values. European Journal of Pharmaceutics and Biopharmaceutics 1997;43:215–242.

[239] Wright JC, Hoffman AS. Historical Overview of Long Acting Injections and Implants. In: Wright JC, Burgess DJ, editors. Long Acting Injections and Implants. Advances in Delivery Science and Technology. Boston, MA: Springer US, 2012. pp. 11–24. doi:10.1007/978-1-4614-0554-2_2.

[240] Wu XS, Wang N. Synthesis, characterization, biodegradation, and drug delivery application of biodegradable lactic/glycolic acid polymers. Part II: Biodegradation. Journal of Biomaterials Science, Polymer Edition 2001;12:21–34.

[241] Yassen A, Olofsen E, Dahan A, Danhof M. Pharmacokinetic-pharmacodynamic modeling of the antinociceptive effect of buprenorphine and fentanyl in rats: role of receptor equilibration kinetics. J Pharmacol Exp Ther 2005;313:1136–1149.

[242] Yassen A, Olofsen E, van Dorp E, Sarton E, Teppema L, Danhof M, Dahan A. Mechanism-Based Pharmacokinetic-Pharmacodynamic Modelling of the Reversal of Buprenorphine-Induced Respiratory Depression by Naloxone. Clin Pharmacokinet 2007;46:965–980.

[243] Yu S, Zhang X, Sun Y, Peng Y, Johnson J, Mandrell T, Shukla AJ, Laizure CS. Pharmacokinetics of Buprenorphine after Intravenous Administration in the Mouse. Journal of the American Association for Laboratory Animal Science 2006;45:12–16.

[244] Yun M-H, Jeong S-W, Pai C-M, Kim S-O. Pharmacokinetic-Pharmacodynamic modeling of the analgesic effect of bupredermTM, in mice. Health 2010;02:824.

[245] Zaki PA, Jr DEK, Brine GA, Carroll FI, Evans CJ. Ligand-Induced Changes in Surface □-Opioid Receptor Number: Relationship to G Protein Activation? 2000;292:8.

[246] Zhu C, Huang Y, Zhang X, Mei L, Pan X, Li G, Wu C. Comparative studies on exenatide-loaded poly (d,l-lactic-co-glycolic acid) microparticles prepared by a novel ultra-fine particle processing system and spray drying. Colloids and Surfaces B: Biointerfaces 2015;132:103–110.

[247] ZILRETTA (triamcinolone acetonide extended-release injectable suspension. n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/208845s000lbl.pdf. Accessed 4 Jul 2020.

[248] ZOLADEX®(goserelin acetate implant). n.d. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/019726s050s051s052lbl.pdf. Accessed 2 Jul 2020.

[249] Zullian C, Lema P, Lavoie M, Dodelet-Devillers A, Beaudry F, Vachon P. Plasma concentrations of buprenorphine following a single subcutaneous administration of a sustained release formulation of buprenorphine in sheep. Can J Vet Res 2016;80:250–253.

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- Development and characterization of parenteral extended release formulations for small molecules and proteins
- Analytical methodologies: RP-HPLC/UPLC, content by UV, LC-MSMS, XRPD, *in vitro* dissolution, particle size analysis, osmolality, protein quantification assays
- Lyophilization, sterilization, physicochemical formulation stability, purity methods, shelf life
- *In vivo* mouse studies with pharmacokinetic and pharmacodynamic analysis
- Market analysis, collaboration with pharmaceutical industry
- Supervision of pharmaceutical bachelor students during practical training (Liquid Sterile Dosage Forms)
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Research scholar at the College of Pharmacy,
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Publications

1. Schreiner V, Detampel P, Jirkof P, Puchkov M, Huwyler J. Buprenorphine loaded PLGA microparticles: Characterization of a sustained-release formulation. Journal of Drug Delivery Science and Technology. 2021 Jun 1;63:102558.

2. Schreiner V, Durst M, Arras M, Detampel P, Jirkof P, Huwyler J. Design and in vivo evaluation of a microparticulate depot formulation of buprenorphine for veterinary use. Scientific Reports. 2020 Oct 14;10(1):1–14.

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