Animal Study

Development of an Effective Topical Liposomal Formulation for Localized Analgesia and Anti-inflammatory Actions in the Complete Freund's Adjuvant Rodent Model of Acute Inflammatory Pain

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Background: Peripheral opioid receptor targeting has been well established as a novel target in clinical pain management for acute and chronic peripheral inflammatory pain. The physiochemical properties of the peripheral mu-opioid receptor agonist, loperamide HCl, limit the use of the free drug as an analgesic or anti-inflammatory agent, particularly for dermal delivery across intact skin.

Objective: Our objective was to manufacture an effective topical formulation containing loperamide using liposomal delivery that would allow loperamide to produce analgesia and anti-inflammatory effects, by penetrating the epidermis to reach peripheral opioid receptors within the dermis of intact skin.

Study Design: A randomized, double blind, controlled animal trial.

Methods: Thirty-five adult male Wistar rats (200 – 250 g) were randomly divided into 5 groups: loperamide HCl-encapsulated liposomal gel, naloxone methiodide + loperamide HCl-encapsulated liposomal gel, free loperamide gel, empty liposomal gel, and 1% diclofenac gel (Voltaren®). Diclofenac gel was used as a positive control as it is clinically used as a topical analgesic and anti-inflammatory drug. Animals received an intraplantar injection of 150 μl Complete Freund's Adjuvant (CFA) into the right hindpaw and experiments were performed 5 days post-CFA injection, which corresponded to the peak inflammatory response. All manufactured formulations were applied topically on both hind paws twice daily, whereas Voltaren gel was applied 3 times a day in accordance with the manufacturer's instructions. The dose administered was 50 μl, which equated to 0.4 mg of loperamide HCl for the loperamide HCl treatment groups (low dose). Naloxone methiodide (1 mg/kg) was administered via intraplantar injection, 15 minutes prior to application of loperamide HCl-encapsulated liposomal gel to determine opioid receptor dependent activity. An investigator blinded to the treatment administered assessed time course of the antinociceptive and anti-inflammatory effects using a paw pressure analgesiometer and plethysmometer, respectively.

Results: Application of loperamide HCl in a liposomal gel formulation exerted analgesic and anti-inflammatory effects exclusively in peripheral painful inflamed tissue. This formulation produced highly significant analgesic and anti-inflammatory effects over the 48-hour time course studied following topical administration in rats with CFA-induced inflammation of the paw. As expected, the diclofenac gel group showed significant antinociception over the duration of the study; however, this effect was lower in comparison to the loperamide HCl liposomal gel formulation. All other control groups showed no significant antinociceptive effects. In addition, all control groups (1% diclofenac gel, free loperamide gel, and empty liposomal gel) did not demonstrate a significant change in paw volume over 48 hours.

Limitations: *In vivo* studies were performed in the well-established rodent model of acute inflammatory pain. We are currently studying this approach in chronic pain models known to have clinical activation of the peripheral immune-derived opioid response.

Conclusions: The study demonstrates that topically applied loperamide encapsulated within liposomal systems has improved therapeutic efficacy over conventional formulations for the local treatment of acute peripheral inflammatory pain conditions where the skin has remained intact. Once in the inflamed peripheral tissue, loperamide provides analgesic and anti-inflammatory effects in a similar manner to peripheral endogenous opioids. This preparation optimises the retention of drug at the site where action is required.

Key words: Pain, inflammation, opioids, loperamide, liposomes, topical drug delivery, peripheral opioid receptors

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Musculoskeletal conditions resulting in peripheral inflammatory pain are prevalent in the community. Topical delivery of drugs is a convenient and well-accepted route of administration for patients with musculoskeletal injuries. Typically, topical pharmacological management of such conditions involves the use of gels containing nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac and ibuprofen. Diclofenac gels are often used for acute musculoskeletal sprains and strains (e.g., sporting injury or back pain) and for chronic conditions such as osteoarthritis in the aging population. For effective treatment, these gels are applied frequently each day and, although designed for a local effect, NSAIDs may also be absorbed into the systemic circulation. Adverse reactions that have been reported include minor dermatitis and pruritus at the site of application (1), to more serious conditions such as gastrointestinal (GI) ulcers and cardiovascular complications when systemic absorption occurs, particularly in the older population (2).

Peripheral opioid receptor targeting provides an alternative therapeutic approach to reduce peripheral inflammatory pain. Peripheral opioid receptor mediated analgesia has been amply demonstrated in patients with various types of pain (e.g., in chronic rheumatoid arthritis (RA) and osteoarthritis (OA), oral mucositis, bone pain, complex regional pain syndrome, and after dental, laparoscopic, urinary bladder, and knee surgery) (3). Peripheral mechanisms of opioid analgesia are well established in the literature (4-6). One of the most extensively studied and most successfully used applications is the intra-articular injection of morphine into inflamed knee joints, which is now established in routine clinical practice (7,8). Peripheral opioids have also been shown to have anti-inflammatory activity by interfering at different stages in the cascade of proinflammatory events in peripheral tissues compared to current anti-inflammatory drugs (6). The anti-inflammatory activity of peripheral opioids has only recently been studied, with results showing potentially a variety of complex regulatory activities in various tissues of the body (3). For example, peripherally acting opioids may induce anti-inflammatory effects as well as analgesia through their opioid receptor mediated actions on neuronal cells and through prevention of vesicular release of neuropeptides or cytokines (e.g., noradrenaline and substance P) (6).

Dermal drug delivery is a complicated process due to the complex nature of the skin. There are 3 main layers of the skin: the outermost epidermis, the dermis, and then the subcutaneous layer. The superficial layer of the epidermis is known as the stratum corneum and is responsible for the majority of the barrier characteristics of the skin. The dermis is primarily composed of connective tissue and it is within this layer that the most superficial free nerve endings and peripheral opioid receptors are located that are associated with pain responses. Topically applied opioids, in particular with morphine, have already been employed as a way of instigating peripheral nociception, particularly for burn injuries or skin ulcers (9-11). In both burns and ulcers the barrier of the skin is impaired, therefore topically applied opioids have access directly to the site of inflammation. However in conditions where the skin remains intact, drug delivery is more complicated.

Loperamide is currently the only peripheral mu-opioid receptor agonist on the market and has a long history of safety and no propensity for abuse. Opioids such as morphine are highly controlled drugs and have significant central opioid-mediated adverse effects and abuse potential, which would limit their chronic and translational use in patients. Loperamide is used extensively to treat diarrhoea due to its effective effect on intestinal motility and secretion, and is used off-label for topical pain relief on open wounds. Unfortunately the physiochemical properties of loperamide limits the use of the free drug as an analgesic or anti-inflammatory agent as it binds to lipids within membranes instead of moving through them. Its exclusion from the central nervous system (CNS) is apparently due to its active removal by the multi-drug resistance transporter, its high affinity to lipid membranes, and its ability to decrease surface tension (12-15). This contributes to its accumulation in membranes and subsequent lack of systemic absorption (12,14). After intravenous or oral application loperamide HCl becomes trapped in the liver, kidneys and lungs, or stomach and intestines, respectively (12).

In order for loperamide to produce analgesia and anti-inflammatory effects across intact skin, it must pass through the epidermis and reach the free nerve endings within the dermis. However due to its lipophilic nature, the drug associates within the stratum corneum and cannot permeate further (Fig. 1). Therefore drug delivery and formulation is required to translate its clinical use as a topical analgesic. Encap-

sulating loperamide within liposomes will allow for its passage into the dermis (16,17) (Fig. 1). Liposomal encapsulation of loperamide has previously been utilized to allow loperamide to be delivered to injured tissue following intravenous injection and for the drug to be able to cross the blood brain barrier (18,19). This technique has also been used for the transdermal delivery of other drugs, such as the anti-fungal drug griseofulvin (20), but has not previously been employed to aid skin permeability of loperamide. This study demonstrates that topically applied loperamide encapsulated within liposomal systems has improved therapeutic efficacy over conventional formulations, for the local treatment of acute peripheral inflammatory pain conditions where the skin has remained intact. Once in the inflamed peripheral tissue, loperamide provides analgesic and anti-inflammatory effects in a similar manner to peripheral endogenous opioids. This preparation has the potential to avoid the adverse effects seen with NSAIDS and optimise retention of the drug at the site where action is required.

METHODS

Preparation of Carbopol Gel

The Carbopol gel was prepared by dispersing carbomer 940 NF resin (PCCA, Houston, Texas, USA) in distilled water (44 g), in which glycerol (5 g) was previously added. Three different formulations were evaluated – 0.5% (w/w), 1% (w/w), and 1.5% (w/w). The mixture was stirred until thickening occurred and then neutralized by the drop wise addition of 50% (w/w) triethanolamine to achieve a transparent gel of pH 5.5. Liposomes were mixed into the Carbopol gel by manual stirring for 5 minutes to ensure homogenous dispersion. To determine which gel was to be used in the *in vivo* experiments specific characteristics were compared. Thickness was evaluated by turning the beaker of gel upside down for 5 seconds. The spreadability of the gel was evaluated by spreading the gel onto the hind paws of male Wistar rats. At the same time the rate of absorbency was evaluated by determining the time taken for the gel to no longer be visually present on the skin. These characteristics are important to determine the suitability of a preparation for use as a topical gel.

High Performance Liquid Chromatography (HPLC) Analysis of Loperamide HCl

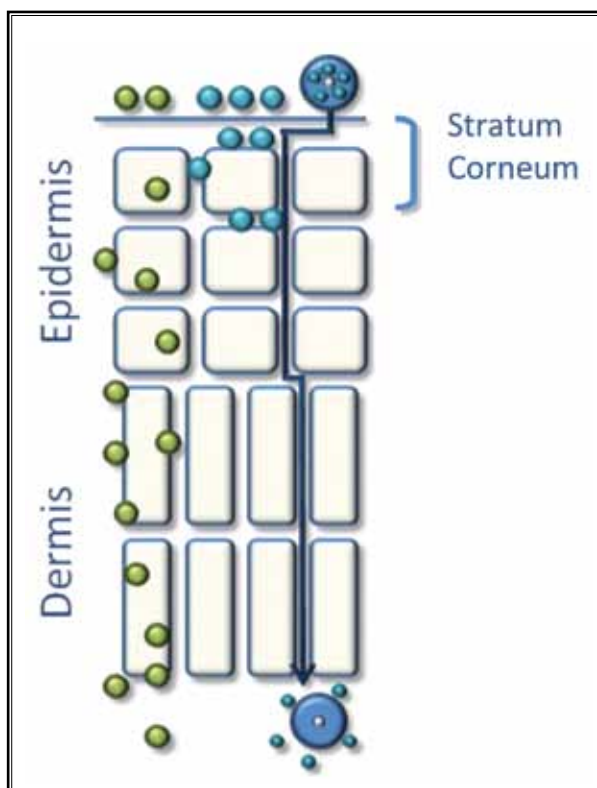The concentration of loperamide HCl was evalu-

Fig. 1. *Skin penetration of non-steroidal anti-inflammatory drugs (NSAIDs) (green circles), free loperamide HCl (small blue circles), and loperamide HCl-encapsulated liposomes (large blue circles) through the dermal layers following topical application. NSAIDs are capable of freely passing through the cells of the epidermis into the dermis, while free loperamide is unable to pass through the stratum corneum. Loperamide HCl-encapsulated liposomes are able to pass between the cells of the epidermis and release the drug in the dermis.*

ated via HPLC (Agilent Technologies 1200 series HPLC system). The HPLC system consisted of a binary pump, autoinjector, column oven, and UV-VIS detector. Separation was performed using a Thermo Scientific BDS Hypersil C18 column (150 x 4.6 mm, 5 µm), which was maintained at a temperature of 25°C and with a detection wave-length of 210 nm. The mobile phase was pumped through the column at a flow rate of 1.5 mL/min and consisted of 5% isopropanol, 50% acetonitrile, and 45% buffer (0.05M NaH_2PO_4 pH 4.5). Data was integrated using Agilent Chemstation software. All chemicals and solvents were of at least analytical grade. Calibration curves were established by plotting the standard concentrations of loperamide HCl versus the area under the curve. Stock solutions were prepared by dissolving 1 mg of loperamide HCl

in 1 mL of ethanol and 5 mL of distilled water (200 µg/mL). A dilution series was then prepared (200µg/mL, 100µg/mL, 50µg/mL, 25µg/mL, 10µg/mL, 5µg/mL, 2.5µg/mL, 1µg/mL, and 0.5µg/mL). Standard curves were prepared from dilutions of 3 independent stock solutions. Samples were then analyzed via HPLC, and the area under the curve (AUC) from the HPLC output was plotted against the loperamide concentration to develop the standard curve.

Preparation of Liposomes

Liposomes were prepared using the dried lipid film hydration method. Different quantities of L-α-phosphatidylcholine (EPC; Avanti Polar Lipid, Alabama, USA) were co-dissolved with 4 mg of cholesterol (Sigma-Aldrich, Sydney, Australia) in 6 mL of chloroform:methanol (2:1, v/v) solution in a 50 mL round bottomed flask, to attain a phospholipid:cholesterol molar ratio of 1:2 (16 mg EPC), 1:4 (32 mg EPC), and 1:7 (56 mg EPC). For the loperamide-encapsulated liposomes, 4 mg of loperamide HCl (Sigma-Aldrich, Castle Hill, NSW, Australia) was also added to the flask. The solution was then dried by rotary evaporation under reduced pressure (100 mbar, 10 min, 37°C). The thin lipid film was then rehydrated under agitation with 1 mL of phosphate buffered saline (PBS; pH 6.5) in a water bath at 37°C. The resultant multilamellar liposomes were then reduced in lamellarity and size via probe sonication (60 amps, 10 mins, 37°C). The size distribution of the liposomal dispersion was determined by dynamic laser light scattering (Zetasizer Nano STM, ATA Scientific, Taren Point, New South Wales). Unencapsulated drug was removed from the liposome suspension using Slide-A-Lyser dialysis cassettes with a 10 kDa MWCO (Thermo Fisher Scientific, Scoresby, Victoria) at 4°C. Encapsulation efficiency (EE%) was determined by disrupting the vesicles with ethanol and evaluating loperamide HCl concentration using HPLC. Liposomes were stored at 4°C and were used within 7 days. Our laboratory has previously confirmed that the liposomes are stable in size, polydispersity, and loperamide concentration over this time period. All chemicals and solvents were of at least analytical grade.

Drug Release Assay of Loperamide HCl Encapsulated Liposomes

Loperamide HCl has limited solubility in water. To ensure that the concentration of loperamide in solution did not reach saturation point and thus affect the assessment of true release of loperamide from liposomes, a modified release assay was performed under sink conditions. Three different formulations were compared containing phospholipid:cholesterol molar ratios of 2:1, 4:1, and 7:1. Loperamide HCl-encapsulated liposomes (50 µl) were dispersed in 1 mL of 0.5% (w/w) Carbopol gel base and this was then placed in a dialysis bag (MWCO 10 kDa; Thermo Fisher Scientific, Australia) with 9 mL of PBS. For the control, 4 mg of loperamide HCl was dissolved in 200 mL of PBS and 10 mL of this solution (equivalent to 200 µg of loperamide HCl) was placed in a dialysis bag. The dialysis tubing was then suspended in a beaker containing 40 mL PBS (pH 6.5) at 37°C on a magnetic stirring plate. Samples of 200 µl were taken at 10 min, 15 min, 30 min, 60 min, 90 min, 2 h, 3 h, 4 h, 6 h, 8 h, and 24 h. The same volume of fresh PBS buffer at the same temperature was added immediately to maintain constant release volume. All experiments were conducted in triplicate with 3 independent samples. Samples were analyzed using HPLC and compared to the standard curve to determine the concentration of loperamide HCl. The loperamide HCl release percentage was obtained according to: *Drug release (%) = (Dt/D0) x 100%*, where Dt and D0 indicate the amount of drug released from the liposome suspension at certain intervals and the total amount of drug in the liposome suspension, respectively. At the end of the study, the liposome samples were recovered from the dialysis system, and lysed with ethanol for analysis of loperamide HCl content by HPLC.

Gel Formulations for *In vivo* Study

Liposomes were manufactured following the same method above with 96 mg EPC, 24 mg cholesterol (molar ratio of 2:1) and 24 mg loperamide HCl was dissolved in 6 mL of chloroform:methanol (2:1, v/v). To rehydrate the lipid film 1940 µl PBS and 60 µl of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen, Victoria, Australia) was used. DiI was added to tag the liposomes pink so that an even dispersion in the gel could be visually gauged. The liposomes were sized to 100 nm by probe sonication. In order to prevent a decreased viscosity of the gel, superfluous liquid was removed from the liposome suspension. The initial volume was 2 mL and this was spun down in an ultrafiltration centrifuge tube (Thermo Fisher Scientific, Scoresby, Australia) at 2500 rpm for one hour to achieve a final volume of 760 µl. Encapsulation efficiency was analyzed via

HPLC. The liposome suspension was then added to 2.25 mL 0.5% (w/w) Carbopol gel which equated to a loperamide HCl concentration of approximately 8 mg/mL. Empty liposomes were made using the same method as above, without the addition of loperamide HCl and 750 μl of this solution was added to 2.25 mL 0.5% (w/w) Carbopol gel. The free drug gel was manufactured by the addition of 16 mg of loperamide HCl to 2 mL 0.5% (w/w) Carbopol gel in order to contain the same concentration of loperamide as the liposomal formulations. The diclofenac gel was a commercially produced one, containing 1% (w/w) diclofenac sodium (Voltaren).

Acute Peripheral Inflammatory Pain Model

Male Wistar rats (6 – 8 weeks; University of Newcastle, Central Animal House) were housed under control conditions (12 hour light-dark cycles, 22°C, 60% humidity) in groups of 2 – 3, with free access to food and water on recycled paper pellet bedding. Rats were given a minimum of 5 days to acclimatise to the housing conditions. After this period rats were anesthetized via brief exposure to 2% isoflurane (Abbot, Cronulla, Australia) before receiving an intraplantar injection into the right hind paw of 150 μl Complete Freund's Adjuvant (CFA; Sigma-Aldrich, Sydney, Australia). CFA is composed of attenuated mycobacterium tuberculosis which activates the innate immune response, leading to inflammation.

Assessment of Antinociceptive and Anti-Inflammatory Efficacy

Experiments were conducted 5 days post-CFA injection, as this time point corresponds to the peak inflammatory response. Baseline measurements were taken prior to administration of the topical formulations. The rats were divided into 5 experimental groups consisting of 6 rats in each group based on treatment: loperamide HCl-encapsulated liposomal gel, loperamide HCl-encapsulated liposomal gel + naloxone methiodide, free loperamide gel, empty liposomal gel, and diclofenac gel (Voltaren). Naloxone methiodide (peripherally selective mu-opioid receptor antagonist) (1 mg/kg; Sigma-Aldrich, Sydney, Australia) was administered via intraplantar injection, 15 minutes prior to application of loperamide HCl-encapsulated liposomal gel to determine opioid receptor dependent activity. All manufactured formulations were applied twice daily (0 h, 10 h, 24 h, and 34 h); Voltaren gel was applied 3 times a day (0 h,

6 h, 10 h, 24 h, 30 h, and 34 h) in accordance with the manufacturer's instructions. Fifty microliters of each formulation was applied to each paw, which is equivalent to 0.4 mg loperamide (low dose). A Band-Aid (Johnson & Johnson) was wrapped around each paw before they were returned to their cage to allow the formulation contact time with the skin. PPT was assessed at 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 24 h, 26 h, 28 h, 30 h, 32 h, 34 h, and 48 h. Paw volume testing was conducted at 24 h and 48 h. The order of contralateral and ipsilateral paw testing was alternated to prevent order effects for PPT, and triplicate measurements were recorded then averaged. Paw volume was ascertained by the use of a rat plethysomometer (Ugo Basile, Comerio, Italy). This involved the placement of the hind paws into the displacement cell and the plethysomometer measures the displacement of water and interprets this as the volume of the paw. PPT was used to approximate hyperalgesia and was measured by a rat analgesiometer (Ugo Basile, Comerio, Italy). This method involves the placement of a blunt probe, which is connected to a sliding weight scale, on the plantar surface of the paw. The weight on the rats paw is increased, either to a maximum ethical load of 250 grams or until the rat flinches.

Statistical Analysis

All data are expressed as means ± standard error of the mean (SEM) or standard deviation (SD). Comparisons were made using paired student t-test with Bonferroni analysis for parametric data. Differences were considered significant when $P < 0.05$.

Results

Characterisation of Carbopol Gel Base

An important consideration in the manufacture of a topical formulation is the gel base that is to be utilized. The concentration of the gelling agent

Table 1. *Properties of gels with different Carbopol concentrations.*

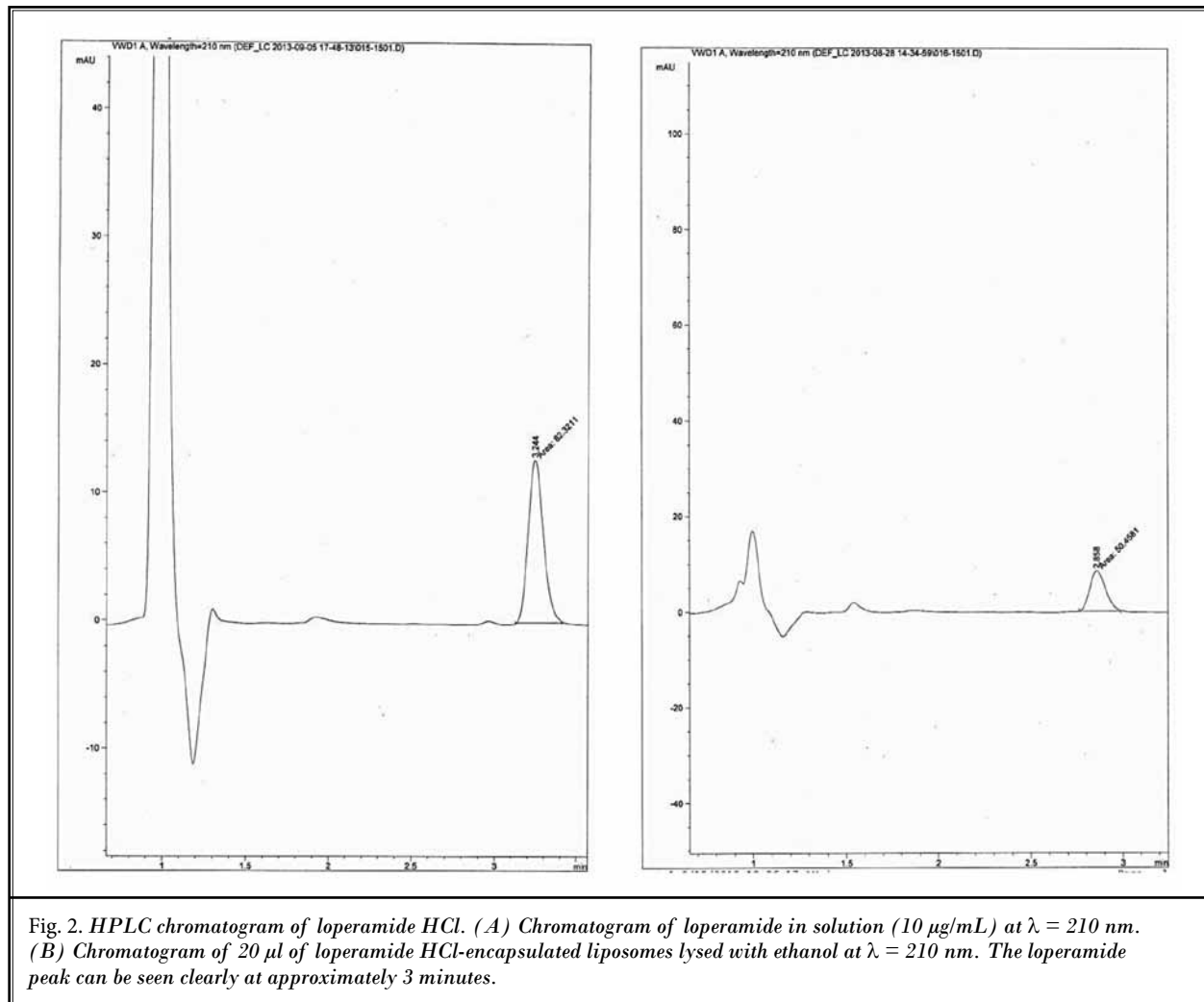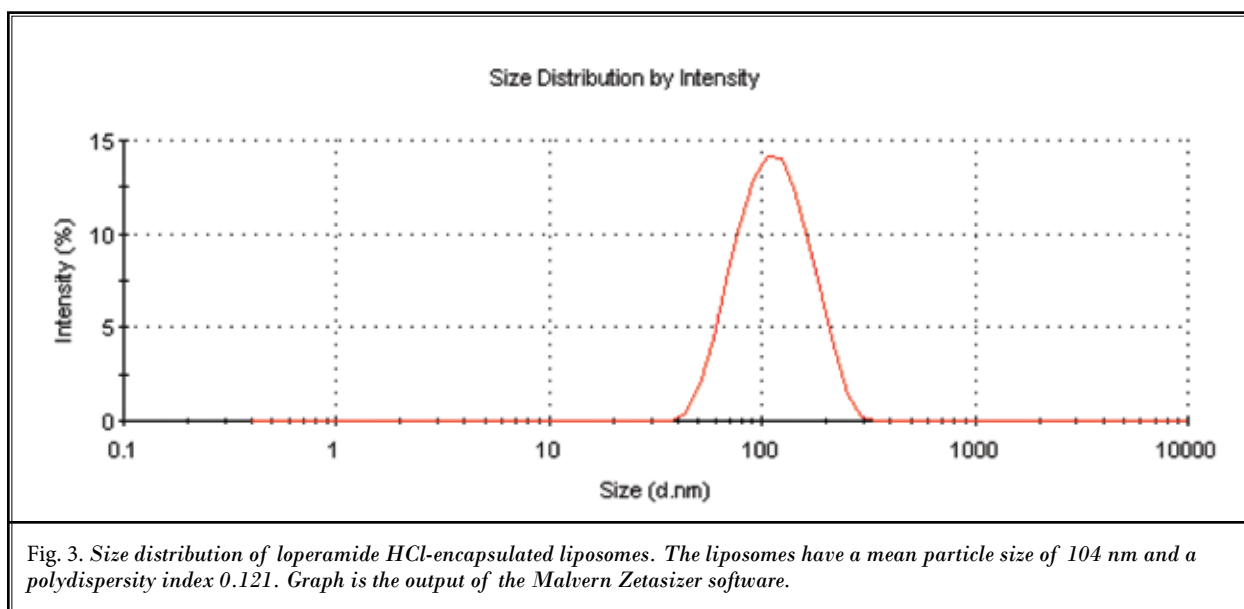|	**0.5% (w/w)**	**1% (w/w)**	**1.5% (w/w)**
Thickness	Does not drip	Does not drip	Does not drip
Absorbency	Very quickly absorbed	Quickly absorbed	Slowly absorbed
Spreadability	Easily spread	Easily spread	Less spreadable (sticky)

Fig. 2. *HPLC chromatogram of loperamide HCl. (A) Chromatogram of loperamide in solution (10 µg/mL) at λ = 210 nm. (B) Chromatogram of 20 µl of loperamide HCl-encapsulated liposomes lysed with ethanol at λ = 210 nm. The loperamide peak can be seen clearly at approximately 3 minutes.*

used determines the characteristics of the gel – a low concentration results in simple gels with a low consistency, whereas higher concentrations lead to more viscose gels that may hinder the production of a gel with a uniform drug distribution. Different Carbopol concentrations were compared in order to determine the most appropriate formulation. The thickness, skin absorbency, and spreadability of 0.5% (w/w), 1% (w/w), and 1.5% (w/w) Carbopol gels were determined (Table 1). Viscosity of the Carbopol gels increased with concentration, while spreadability and absorbency decreased. A concentration of 1.5% (w/w) generated a gel that was too thick; it was difficult to spread upon the skin and left a residue. Conversely, gels manufactured with Carbopol concentrations of 0.5% and 1% had suitable characteristics.

Evaluation of Loperamide HCl Concentration via HPLC

A typical chromatogram for loperamide HCl can be seen in Fig. 2A. The retention time (i.e., length of time the testing compound remains bound to the HPLC column) for loperamide HCl is approximately 3 minutes. Fig. 2B depicts a typical chromatogram for a sample of loperamide HCl that has been released from liposomes using ethanol. This figure demonstrates that the retention time for loperamide is unchanged by the presence of lipids and no peaks from the constituents of the liposomes affect peaks around the 3 minute mark. In order to determine the concentration of loperamide in HPLC analyzed samples a standard curve was generated. Known concentrations of loperamide were analyzed via HPLC. This equation

Fig. 3. *Size distribution of loperamide HCl-encapsulated liposomes. The liposomes have a mean particle size of 104 nm and a polydispersity index 0.121. Graph is the output of the Malvern Zetasizer software.*

was utilized in later experiments to calculate the concentration of loperamide in samples.

Determination of Liposomal Dispersion Properties

Loperamide HCl-encapsulated liposomes were sized to approximately 100 nm, with a polydispersity index (PDI) of less than 0.3. A low PDI (< 0.3) signifies that the mean particle size is an adequate indicator of the size variance in the entire sample. Fig. 3 represents a typical output from the zetasizer. The bell curve demonstrates a normal distribution of particle size, with a mean particle size of 104 nm and a polydispersity index of 0.121. This method resulted in liposomes with an encapsulation efficiency of 92.74 ± 4.08% which equated to an average of 3.71 ± 0.16 mg of loperamide HCl being encapsulated in each milliliter of liposomes. Encapsulation efficiency was determined via HPLC analysis of ethanol lysed liposomes and reporting this value as a percentage of the original 4 mg of loperamide HCl used in the manufacture of the liposomes.
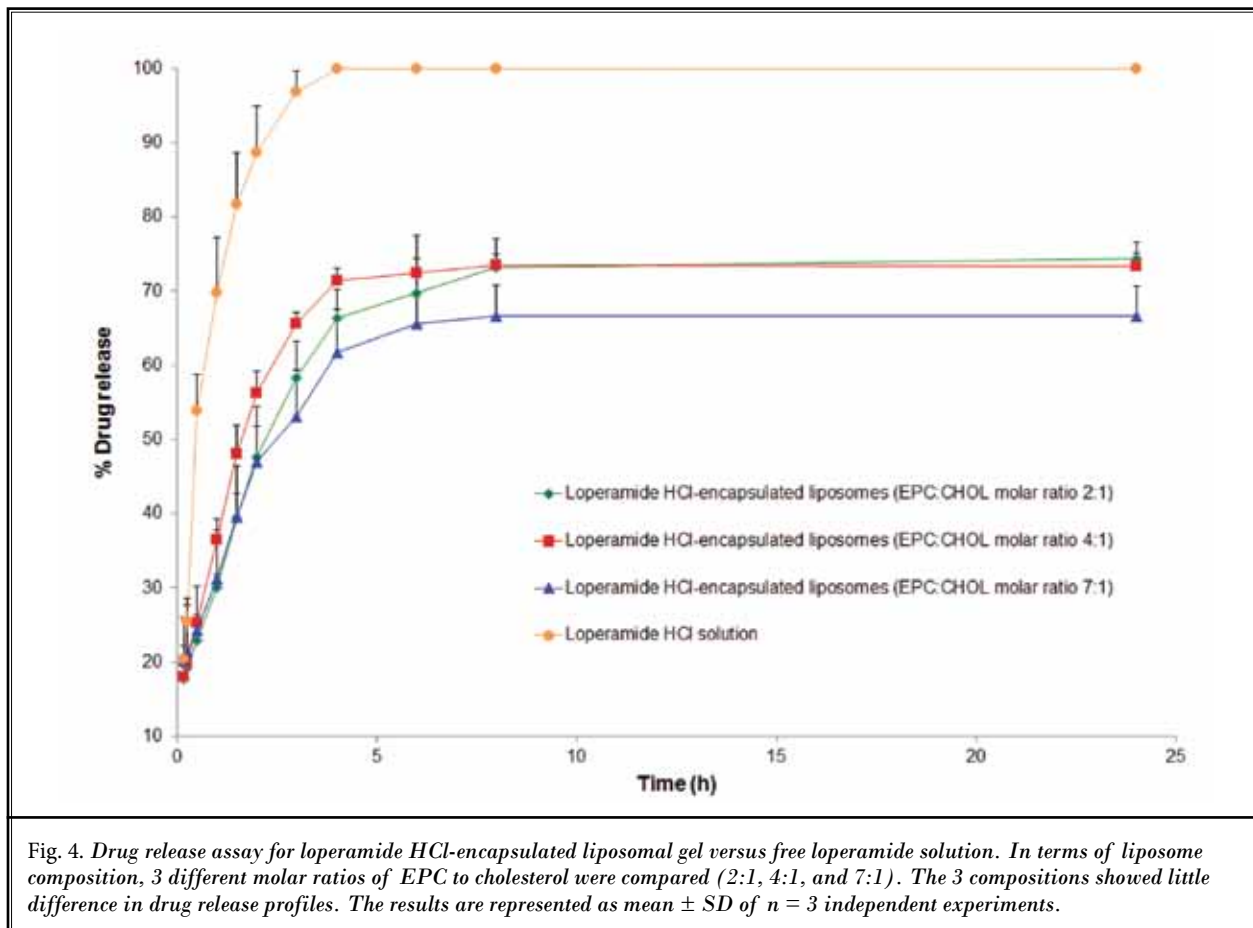
Stability of Loperamide HCl-Encapsulated Liposomes

Fig. 4 shows the drug release profile of loperamide from liposomes composed of EPC:cholesterol with a molar ratio of 2:1, 4:1, and 7:1, as well as free loperamide dissolved in PBS (pH 6.5). A pH of 6.5 was used as this approximates the pH of the skin. The max-

imum solubility of loperamide HCl at pH 6.5 is 4 mg in 200 mL of PBS. Each solution was suspended in PBS in dialysis tubing, which was then placed in a beaker of PBS. Aliquots of the release medium were taken at set time intervals and analyzed for drug content. Fig. 4 demonstrates that the percentage of free loperamide HCl to pass through the dialysis membrane reached 100% in the first 5 hours. This demonstrates that loperamide was able to pass freely across the dialysis membrane. The release profile of the liposomes exhibited a rapid release in the first 3 hours that reached around 50% of the encapsulated loperamide HCl. The release then slowed, to plateau at around 70% release by the 8 hour time point. There was little difference between the loperamide release profiles of the 3 different liposome compositions.

Acute Peripheral Inflammatory Pain Model

Administration of CFA resulted in pronounced unilateral hyperalgesia and oedema in the inoculated ipsilateral hind paw. PPT and paw volume were measured prior to and after intraplantar CFA injection (n = 30). The mean PPT prior to CFA injection was 226 ± 6.64 g for the contralateral hind paw and 215.3 ± 6.98 g for the ipsilateral hind paw. The mean paw volume for the contralateral hind paw was 1.44 ± 0.04 mL and 1.50 ± 0.03 mL for the ipsilateral hind paw. On day 5 post-CFA injection, the inoculated ipsilateral hind paw had a PPT of 97.3 ± 6.88 g, and a paw volume of 2.66 ± 0.05 mL. CFA injection generated a significant inflam-

Fig. 4. *Drug release assay for loperamide HCl-encapsulated liposomal gel versus free loperamide solution. In terms of liposome composition, 3 different molar ratios of EPC to cholesterol were compared (2:1, 4:1, and 7:1). The 3 compositions showed little difference in drug release profiles. The results are represented as mean ± SD of n = 3 independent experiments.*

matory response in terms of PPT and paw volume (P < 0.0001). Analysis of the contralateral hind paw gave no statistical difference pre- and post-inoculation, with a mean PPT of 232 ± 4.09 g and a paw volume of 1.50 ± 0.05 mL $(P > 0.05)$. These results demonstrate an increase in paw volume of over 60% and a 2-fold decrease in PPT after intraplantar injection with CFA.
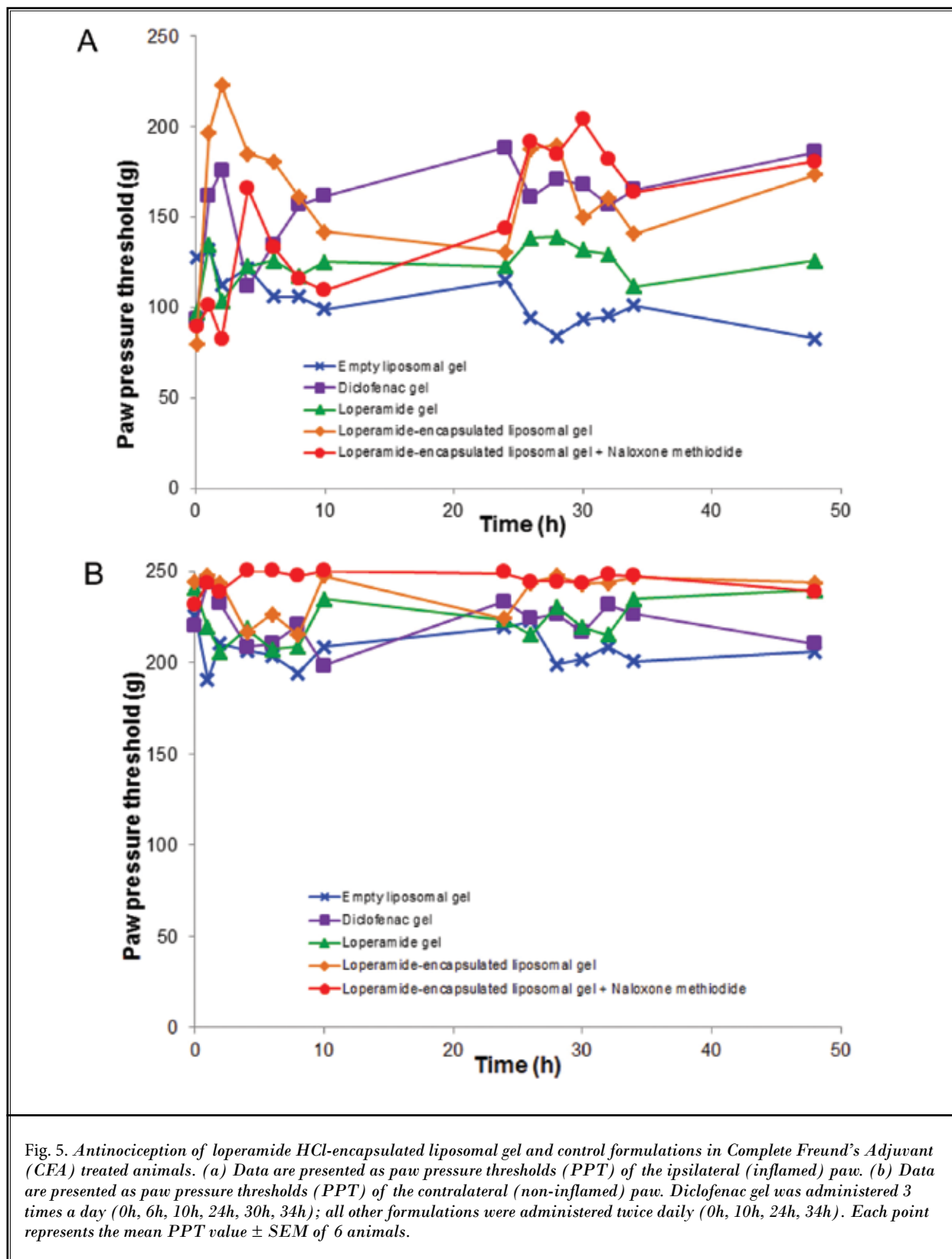
Time Course of the Antinociceptive Effect

PPT was used as a measure of the antinociceptive effect of loperamide HCl-encapsulated liposomal gel. This was evaluated over 48 hours to determine the peak and duration of response of the liposomal formulation in comparison to the control groups. Figs. 5 and 6 indicate that application of loperamide HCl-encapsulated liposomal gel to the ipsilateral (inflamed) paws of CFA-treated rats resulted in antinociception across the 48 hours testing period when compared to the control groups (i.e., the rats withstood a greater mass before flinching; P < 0.01). The peak response for

the loperamide HCl-encapsulated liposomal gel was identified between 2 and 4 hours after each dose was applied. A peak value of 222.5 ± 17.69 g was observed in the first 12 hours and 190.0 ± 14.83 g following the last application in the 48 hour testing period. The PPT decreased after the peak response, however still remained significantly higher compared to baseline values (P < 0.05), therefore demonstrating significant antinociceptive effect throughout the duration of the study. Figs. 5 and 6 demonstrate that the empty liposomes and free loperamide gel had no significant antinociceptive effect (P > 0.05). The contralateral, non-inflamed hind paws produced no differences in PPT throughout the 48 hours study, regardless of the treatment group (Fig. 5; P > 0.05).

To examine if the observed antinociceptive effect of loperamide HCl-encapsulated liposomes was due to interaction with peripheral opioid receptors, intraplantar naloxone methiodide was administered 15 minutes prior to the formulation being applied (Figs. 5 and 6). Naloxone

Fig. 5. *Antinociception of loperamide HCl-encapsulated liposomal gel and control formulations in Complete Freund's Adjuvant (CFA) treated animals. (a) Data are presented as paw pressure thresholds (PPT) of the ipsilateral (inflamed) paw. (b) Data are presented as paw pressure thresholds (PPT) of the contralateral (non-inflamed) paw. Diclofenac gel was administered 3 times a day (0h, 6h, 10h, 24h, 30h, 34h); all other formulations were administered twice daily (0h, 10h, 24h, 34h). Each point represents the mean PPT value ± SEM of 6 animals.*
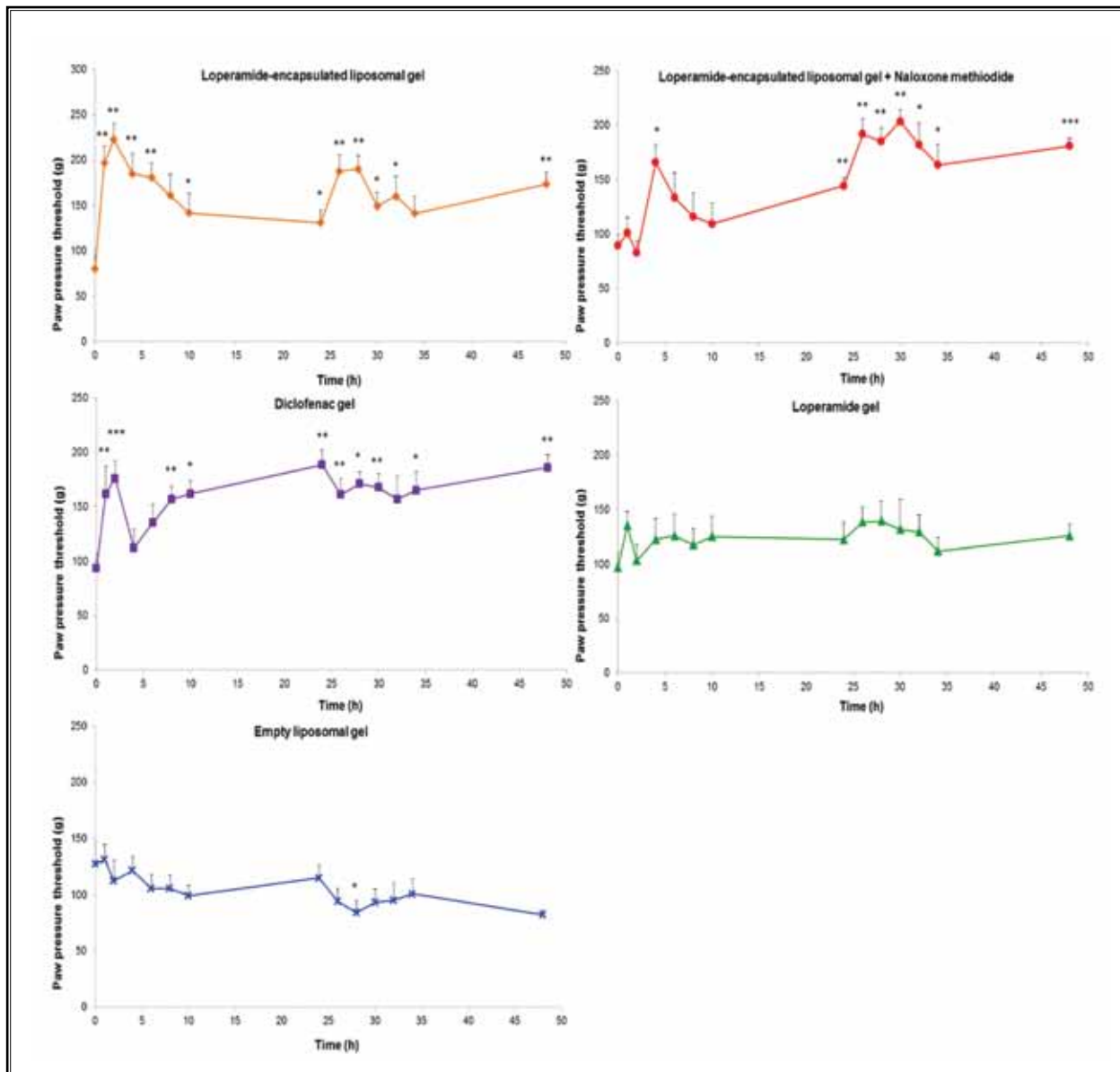
Fig. 6. *Individual graphs of antinociception over time for the different study groups in CFA treated animals. Each point represents the mean PPT value ± SEM of 6 animals. Intergroup differences were assessed using paired student t-test with Bonferroni analysis for parametric data (*P < 0.05, **P < 0.01, ***P < 0.001).*

methiodide was able to block the antinociceptive effect of loperamide over the first 4 hours ($P > 0.05$), which corresponds with the duration of its effect. As the naloxone wore off a peak response was observed at 4 hours, being 165.83 ± 15.7 g which is lower than that observed in the loperamide HCl-encapsulated liposomal gel group not treated with naloxone; thereafter the antinociceptive profile was similar to that for the loperamide HCl-encapsulated liposomal gel group. This confirmed that the

increase in PPT seen was due to the activation of an opioid receptor dependant pathway. Diclofenac gel (Voltaren) was used as a positive control as it is clinically used as a topical analgesic and anti-inflammatory drug. The diclofenac gel demonstrated a significant antinociceptive effect as expected over the duration of the study (Figs. 5 and 6; $P < 0.001$. The peak PPT score was 188.3 ± 13.82 g, which is lower than either peak noted for the loperamide HCl-encapsulated liposome gel relative to baseline.

Time Course of the Anti-inflammatory Effect

Paw volume was used as an indicator of the anti-inflammatory efficacy of the loperamide HCl-encapsulated liposomes formulation, at 0 hours, 24 hours, and 48 hours. No significant difference was noted in the volume of the contralateral (non-inflamed) hind paw throughout the duration of the experiment (Fig. 7; $P > 0.05$). The inflamed hind paws had an average change in volume that approached a 2-fold increase, when compared to the non-inflamed hind paws at baseline testing. This indicates a localized, acute inflammatory response, brought about by administration of CFA. Over the 48 hour testing period, paw volumes of 2.35 ± 0.15 mL and 2.17 ± 0.12 mL were recorded at 24 h and 48 h respectively, for the loperamide HCl-encapsulated liposomal gel as compared to the baseline of 2.77 ± 0.16 mL (Figs. 7 and 8). These results show a significant anti-inflammatory effect for the loperamide HCl-encapsulated liposomal gel ($P < 0.01$). The group administered naloxone methiodide + loperamide HCl-encapsulated liposomal gel also demonstrated a significant decrease in paw volume being 2.50 ± 0.1 mL at 24 h and 2.32 ± 0.08 mL at 48 h, with a baseline of 2.66 ± 0.07 mL (Fig. 8; $P < 0.05$). The group treated with empty liposomes maintained a relatively constant paw volume, while the groups treated with free loperamide gel and diclofenac gel had increased paw volumes over the 48 hour study period (Fig. 8; $P > 0.05$). This indicates that the free loperamide gel and diclofenac gel had no significant anti-inflammatory effect over the duration of the study.

Discussion

We have manufactured a topical formulation utilizing liposomal delivery as a method of enhancing the dermal delivery of the peripheral opioid, loperamide HCl, across intact skin for use in acute and chronic musculoskeletal conditions involving peripheral inflammatory pain. Analgesia following topical administration of opioids has been previously demonstrated. Case studies have reported the effectiveness of morphine when combined with pre-made gels such as IntraSite (Smith & Nephew) for use in skin ulcers. Van Ingen et al (9) reported a case where topical morphine gel was applied to a cutaneous ulcer that had previously been treated unsuccessfully with zinc oil and surgical debridement – the visual analogue scale (VAS; 0 – 100) for pain following topical morphine application was halved from 80 to 40 within 3 days of treatment. Conversely, Welling (10) reported the relative ineffectiveness of topical morphine in burns patients. This may be due to damaged nerve endings, which result in impaired signal transduction or receptor expression. In humans, loperamide has also been used in solution as a mouthwash for analgesia in graft-versus-host related oral pain with positive results (21). Nozaki-Taguchi and Yaksh (11) explored the use of topical loperamide in a rat model of burns injury and showed that thermal hyperalgesia was able to be alleviated following topical loperamide treatment.

We have shown that application of loperamide to dermal sites where skin integrity has not been affected (i.e., peripheral inflammatory pain with intact skin) has no significant analgesic activity. This is due to loperamide having a high affinity for lipid membranes and the ability to reduce surface tension and thus congregates in membranes rather than crossing them, therefore restricting its action to its immediate location of delivery (22). The advantage of this characteristic is that it severely limits the ability of loperamide to cause adverse central opioid-mediated side effects. The disadvantage, however, is that it limits the topical use of this drug to painful conditions associated with open wounds (e.g., burns or ulcers). Additionally, the use of penetration enhancers (e.g., propylene glycol) in the topical formulation base does not readily improve the dermal delivery of loperamide, with *in vitro* studies over-estimating its efficacy *in vivo* (23). Therefore we explored the use of liposomes as a delivery mechanism to enhance the topical permeability of loperamide across painful conditions involving intact skin.

Topical liposomal formulations have been shown to improve the efficacy of therapeutic agents by increasing the local concentrations of the drug they contain at the site of action, as well as reduce excretion (24). For example, liposomal gel formulation of the corticosteroid, prednisolone, demonstrated both enhanced activity and sustained release in comparison to conventional prednisolone gel formulations in a model of rheumatoid arthritis (25). 18β-glycyrrhetic acid, which is used for the treatment of chronic dermatitis, showed increased efficacy and bioavailability following liposomal encapsulation when compared to free gel formulations (26). Likewise, liposomal delivery of topical brucine, a complementary medicine used for arthritic pain, demonstrated enhanced efficacy and bioavailability as well as reduced toxicity following liposomal encapsulation (27). Irfan (28)
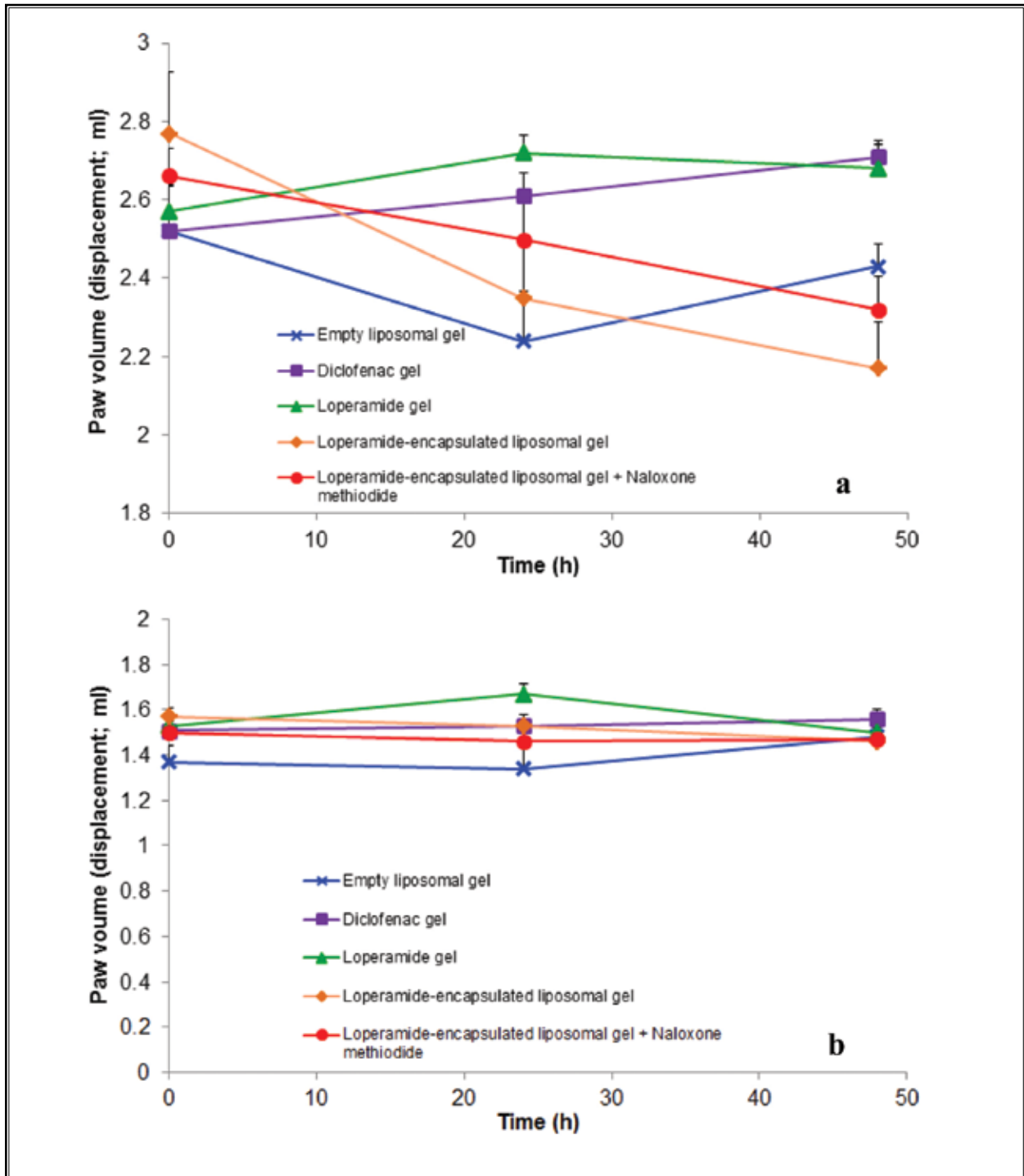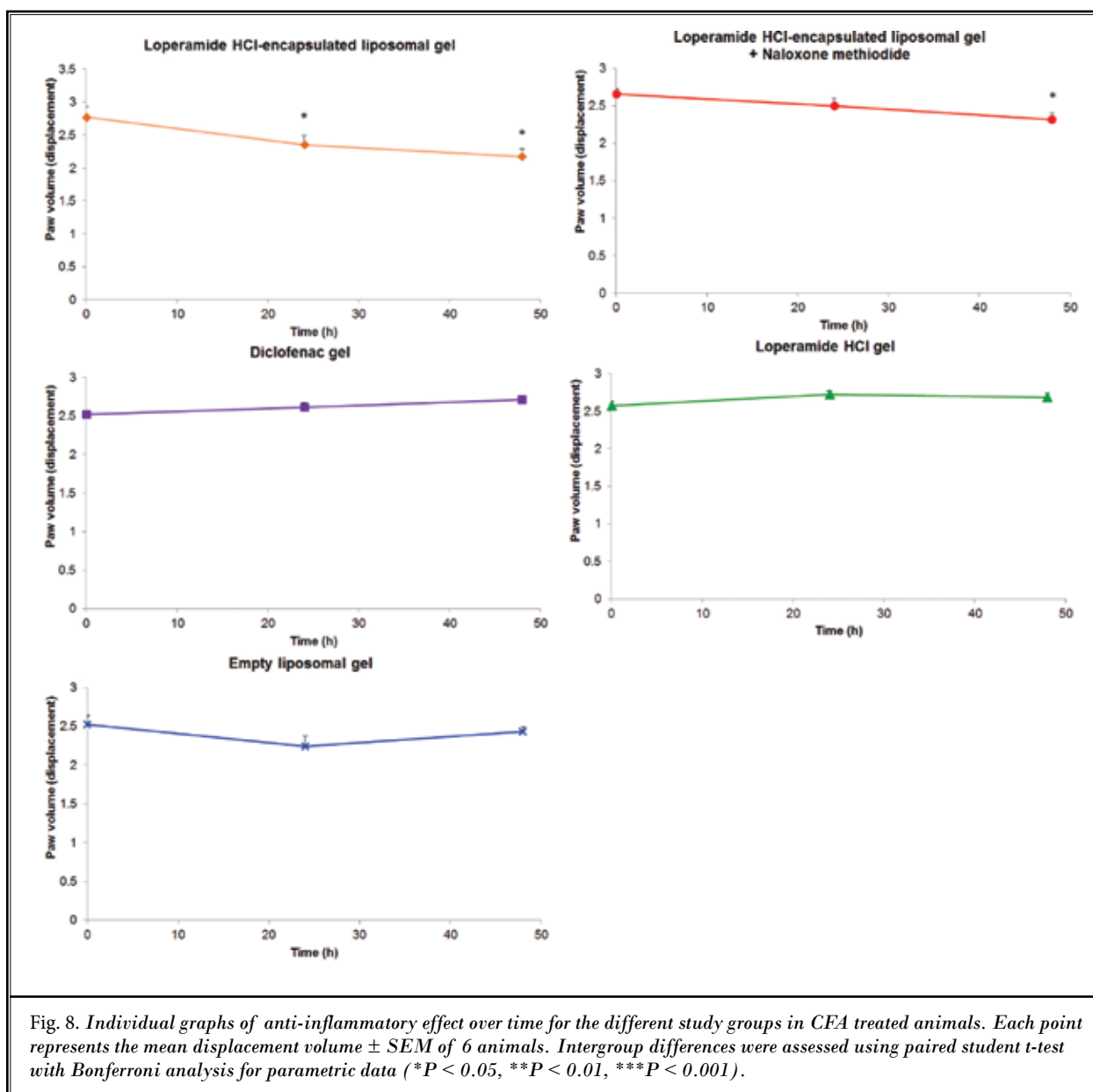
Fig. 7. *Anti-inflammatory effect of loperamide HCl-encapsulated liposomal gel and control formulations in Complete Freund's Adjuvant (CFA) treated animals. (a) Data are presented in terms of paw volume of the ipsilateral (inflamed) paw. (b) Data are presented in terms of paw volume of the contralateral (non-inflamed) paw. Diclofenac gel was administered 3 times a day (0h, 6h, 10h, 24h, 30h, 34h); all other formulations were administered twice daily (0h, 10h, 24h, 34h). Each point represents the mean displacement volume ± SEM of 6 animals.*

Fig. 8. *Individual graphs of anti-inflammatory effect over time for the different study groups in CFA treated animals. Each point represents the mean displacement volume ± SEM of 6 animals. Intergroup differences were assessed using paired student t-test with Bonferroni analysis for parametric data (*P < 0.05, **P < 0.01, ***P < 0.001).*

demonstrated that encapsulation of the lipophilic NSAID, ibuprofen, within a liposomal gel formulation enhanced the skin permeability and stability of the free drug. Furthermore, liposomes are generally considered nontoxic, biodegradable, and nonimmunogenic, as they are composed predominantly of naturally occurring lipids that form the membranes within the body (29). Parnham and Wetzig (30) conducted a study into the toxicology of liposomes composed of phospholipids and cholesterol and reported their

safety for use in pharmaceutical and cosmetic applications. These studies show the applicability of dermal and transdermal liposomal delivery of drugs for peripheral analgesic and anti-inflammatory action.

Characteristics of the liposomal formulation itself determine the efficacy for dermal delivery of encapsulated drugs. For example, phospholipid composition controls liposome stability and drug release. Encapsulation of loperamide within liposomes composed of 1,2-distearoyl-sn-glycero-2-phosphocholine (DSPC)

has been shown to enhance their transport within the blood and prevent loperamide from associating with membranes (19). However, studies have shown that DSPC-based liposomes are unable to permeate the dermal layers of the skin. Conversely, liposomes composed of l-α-phosphatidylcholine (EPC) are able to cross the stratum corneum (31). Therefore EPC was selected for the manufacture of liposomes in our study (32). It has been hypothesized that an alteration in skin lipid bilayer fluidities may be involved in this percutaneous penetration (33). The phase transition temperature of EPC is around 19°C, which means that at temperatures above this there is a conformational change in the phospholipid to a more fluid state. This means that the liposomes will release the encapsulated loperamide once past the upper dermal layers as the temperature approaches core body levels.

Size is another important parameter in determining the passage of liposomes between the cells of the dermal layers. There is some controversy within the literature as to what liposome size is optimal. Sentjurc et al (34) proposed that liposomes smaller than 200 nm are unstable and degrade when in contact with the skin, forming a lipid layer on the skin that prevents any drug or other liposomes from passing. Conversely, Harashima et al (35) demonstrated through kinetic modelling that liposome degradation rates increased parallel with the size of liposomes, thus suggesting that liposomes with a diameter less than 200 nm are more stable. Lasic (36) reported that liposomes in the size range of 50 – 150 nm are optimal for skin permeability, as this range is a compromise between loading efficiency of liposomes (increases with increasing size), liposome stability (decreases with increasing size above an optimal 80 – 200 nm range) and ability to extravasate (decreases with increasing size). Based on these previous findings, a liposome size of approximately 100 nm was selected for our study.

The formulation base used to disperse the loperamide HCl-encapsulated liposomes is another important consideration. The vehicle must have a suitable pH and rheological characteristics, while not causing skin irritations or adversely affecting the stability of the liposomes. Carbopol is a commonly used gelling agent that rarely causes adverse reactions when utilized topically (37). Its safety for both internal and external administration has been determined following thorough toxicological studies (37,38). It is composed of cross-linked carbomer polymers that form a microgel structure. It is this microgel arrangement that makes this gel so suitable for topical application, as the gel can easily reconfigure itself with the movement of the body (37). Furthermore, these gels have exhibited an ability to aid in the stability of liposomes over time (32), and possess a buffering capacity and residue that creates an ideal pH for the liposomes (33). Our study showed that Carbopol gels at a concentration of 0.5% (w/w) and 1% (w/w) were ideal for topical administration. An increase in the Carbopol concentration increased the viscosity of the gel, which adversely affects the uniform distribution of (liposomal) drug dispersion throughout the gel and the application onto the skin. The pH of the different gels were kept constant at pH 5.5, as the viscosity of the gel base is altered by pH and as this pH is optimal for topical application to skin.

The major factors controlling liposome stability and release kinetics are the lipids used in the formulation and the cholesterol concentration within the liposomes. In order to determine the most appropriate ratio of EPC to cholesterol in the structure of the liposomes to be taken into *in vivo* studies, the rate of drug release of loperamide was evaluated. The stability of liposomes and the rate of release of drug *in vitro* is an indicator of the *in vivo* bioavailability of a drug. Phospholipids are the main constituent of liposomes and are the major lipid component of biological membranes. Cholesterol is often included in liposome formulations to stabilize the lipid bilayer by inducing conformation ordering of lipid chains, and to decrease the leakage of encapsulated drug by reducing both bilayer permeability and serum-induced instability of the vesicle structure (39,40). An optimal ratio of cholesterol to EPC will create liposomes that are stable upon the surface of the skin and allow loperamide to be released within the dermis. Our drug release assay demonstrated little difference in terms of drug release between the 3 tested phospholipid:cholesterol molar ratios and as such, the 2:1 ratio was taken into *in vivo* studies as it was the most cost effective to manufacture.

The *in vivo* efficacy of our loperamide HCl-encapsulated liposomal gel was evaluated in the well-established CFA model of acute peripheral inflammatory pain (13,19,41,42). In our study, administration of CFA produced visibly localized redness and swelling in the inoculated paw. Swelling was measured quantitatively through water displacement values of the paws. Average paw volumes for the inflamed paws were significantly increased post-intraplantar injec-

tion of CFA, while values of the non-inflamed paws were unaltered. Hyperalgesia was also observed, localized to the inoculated paw. Paw pressure threshold (PPT) testing was used as a measure of hyperalgesia. Injection of CFA caused a significant decrease in the weight that could be applied to the injected paw, while the PPT remained high in the non-inflamed paws. These experimental results reflect the ability of CFA injection to model an acute peripheral inflammatory response.

Loperamide HCl-encapsulated liposomal gel demonstrated a significant antinociceptive effect when applied to the ipsilateral (inflamed) hind paw. The PPT for this treatment group was significantly higher over the 48 hours study period in comparison to baseline, and was able to be reversed with naloxone methiodide, thus demonstrating an opioid receptor dependent mechanism. The control groups (empty liposomal gel and free loperamide gel) did not demonstrate any antinociceptive effect, which indicate that the efficacy of the loperamide HCl-encapsulated liposomal gel was due to loperamide being released within the dermis of the inflamed tissue in close proximity to peripheral opioid receptors. In the study by Nozaki-Taguchi and Yaksh (11), they showed that thermal hyperalgesia was able to be alleviated following topical loperamide treatment in a burns injury model. Antinociception was only evident after using significantly higher concentrations of loperamide (5 mg per dose), and this dose only had a duration of action of approximately 2 hours; this is in comparison to our study which had 0.4 mg per dose with a peak response at 4 hours. We have previously demonstrated that an intravenous administration of ICAM-1 targeted immunoliposomes containing loperamide HCl was able to produce significant antinociceptive action for over 48 hours following a single dose containing 0.8 mg loperamide HCl (19). Therefore liposomal delivery allows a much lower dose of drug to be administered as well as a more sustained release effect.

The antinociceptive effect of loperamide HCl-encapsulated liposomal gel was comparable to that of the diclofenac gel with regards to PPTs. Topical NSAIDs are currently used as the gold standard for the topical treatment of peripheral inflammatory pain, however, are associated with drug interactions, adverse reactions, and compliance issues. In terms of compliance, diclofenac gels require frequent application up to 4 times a day, which can be an issue in the treatment of acute and chronic musculoskeletal conditions. The

peak of the antinociceptive effect for loperamide HCl-encapsulated liposomal gel was noted 2 to 4 hours after each dose was administered. Twice daily application in this study was sufficient to produce significant antinociception for the liposomal formulation, with a peak greater than that for the diclofenac gel group that involved 3 times daily application. Future testing should explore the efficacy of once daily application, as this would further assist with compliance. Our preclinical study demonstrates that application of loperamide HCl-encapsulated liposomal gel is potentially a more effective analgesic treatment than currently available NSAID-based topical preparations.

Loperamide HCl-encapsulated liposomal gel also had significant anti-inflammatory effects. Analysis of paw volume showed a significant decrease at both 24 hours and 48 hours in comparison to baseline displacement values, even when naloxone methiodide was administered beforehand. As the duration of action of naloxone methiodide is relatively short, we did not expect this to affect the anti-inflammatory response of the loperamide HCl-encapsulated liposomal gel, as anti-inflammatory action is never immediate. This anti-inflammatory effect may be due to an opioid-receptor independent mechanism (3,5,19,43-49). All control groups (1% diclofenac gel, free loperamide gel, and empty liposomal gel) did not demonstrate a significant change in paw volume over 48 hours. Interestingly, our study did not detect any anti-inflammatory effect for the diclofenac gel, which is in contrast to other studies (50). This highlights the potential clinical significance of loperamide HCl-encapsulated liposomes in the management of both hyperalgesia and inflammation associated with peripheral inflammatory pain.

Conclusion

This study indicates the importance of liposome encapsulation to enhancing the antinociceptive and anti-inflammatory efficacy of loperamide HCl following topical administration. This formulation was well tolerated following topical administration in a well-established rodent model of acute peripheral inflammatory pain, and was more effective than diclofenac in terms of reducing hyperalgesia and inflammation. Although the antinociceptive and anti-inflammatory testing methods used are well-established in the literature, future studies may consider using additional methods (e.g., assessing thermal hyperalgesia or using an electronic von Frey anesthesiometer to assess

mechanical allodynia). It will also be of much interest to confirm the mechanism of the anti-inflammatory effect of the loperamide HCl-encapsulated liposomal gel. This topical formulation may potentially be used in the management of acute painful inflammatory conditions (e.g., musculoskeletal sprains and strains) and chronic painful inflammatory conditions (e.g., osteoarthritis). Further studies have been planned to assess the formulation in *in vivo* chronic pain models and assessing the tolerability of the formulation over a longer duration. These results are encouraging in the progress towards novel, effective, and safe analgesic and anti-inflammatory therapies that avoid the serious central side effects of opioids (e.g., respiratory depression, sedation, tolerance, and dependence) and those of nonsteroidal anti-inflammatory drugs (e.g., GI ulcers or bleeding, hypertension, renal impairment, cardiovascular complications, rash, and bronchospasm).

Acknowledgments

Ethics of animal experiments

The experiments were approved by the University of Newcastle animal ethics committee.

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