DRV Liposomal Bupivacaine: Preparation, Characterization, and *In Vivo* Evaluation in Mice

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Purpose. To evaluate the dehydration-rehydration technique to prepare a formulation of liposomal bupivacaine, and to assess its analgesic efficacy.

Methods. Bupivacaine hydrochloride (BUP) was encapsulated into dehydration-rehydration vesicles (DRV) of varying phospholipid (PL) compositions. Two bilayer-forming phospholipids were used, the "fluid" dimyristoyl-phosphatidylcholine and the "solid" distearoyl-phosphatidylcholine (DSPC), with 20 or 40 mol% cholesterol, in the presence of bupivacaine at a 1.28 or 0.64 BUP/PL mole ratio. After rehydration, drug/lipid ratios were determined. The formulation with the highest drug/lipid ratio (DSPC/cholesterol in an 8:2 mole ratio prepared in the presence of bupivacaine in a 1.28 BUP/PL mole ratio) was adjusted to a final bupivacaine concentration of 3.5% or 0.5%. The duration of skin analgesia after subcutaneous injection in mice produced by these formulations was compared with the conventional administration of a plain 0.5% solution of BUP. In addition, the concentration of residual bupivacaine at the injection site was followed for 96 h.

Results. The relatively low organic solvent/aqueous phase and membrane/aqueous phase partition coefficients, together with liposomal trapped volume and BUP/PL mole ratio, indicated that most of the drug was encapsulated in the intraliposome aqueous phase of the DRV. The DSPC/cholesterol 8:2 mole ratio had the best drug encapsulation (BUP/PL = 0.36). Compared to plain BUP, these BUP-DRV produced significant prolongation of analgesia, which is explained by longer residence time of the drug at the site of injection.

Conclusions. Bupivacaine-DRV may have a role in achieving safe, effective, and prolonged analgesia in humans.

KEY WORDS: analgesia; drug delivery; drug/lipid ratio; local anesthetics.

INTRODUCTION

Long-acting local anesthetic formulations hold great promise for the management of acute pain, as long-lasting analgesia could be achieved with a single dose administered after surgery or trauma. Liposomal bupivacaine (BUP) formulations prolong analgesic duration in animals (1–4) and humans (5–7). The slow release of drug from the liposomal depot decreases the potential for systemic toxicity, and allows for administration of a greater BUP dose (1,8).

However, before a liposomal local anesthetic product can be used to manage acute pain in patients, many issues need to be resolved. Some critical requirements for a liposomal formulation are reliability and reproducibility in manufacturing and performance, and adequate shelf stability to permit long-term storage. In aqueous media, the lipid constituents are subject to degradation due to oxidation and hydrolysis. Moreover, encapsulated drug may leak from the liposome into the aqueous medium. To date, the liposomal local anesthetics which have been described in the literature are either multilamellar vesicles (MLV) (1,9) or large unilamellar vesicles (LUV) (3). These liposomes are stored in aqueous media, and therefore have limited stability. A freeze-dried formulation of high stability would obviate this problem. Furthermore, the highest drug/lipid ratio reported for these formulations is 0.26 (3). Dehydration-rehydration vesicles (DRV) are liposomes that can be reproducibly prepared at high drug/lipid ratio, stored in a lyophilized state, and rehydrated immediately prior to administration (10). Maintaining the formulations in the dehydrated state greatly reduces the rate of degradation and confers shelf stability (11). This study was designed to evaluate the feasibility of using DRV technology to encapsulate BUP, and to assess analgesic efficacy in a mouse model.

METHODS

Preparation of DRV

BUP hydrochloride (Sigma, St. Louis, MO) was encapsulated into liposomes using a modified DRV technique (12). To achieve the optimal liposomal bupivacaine composition, four parameters were manipulated: bilayer lipid, percent cholesterol (CHOL), amount of BUP, and pH of liposome dispersions during preparation. Two different types of bilayer-forming lipids were used: 1,2-dimyristoyl- *sn*-glycero-3-phosphocholine (DMPC) and distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, Alabaster, AL). These lipids were chosen to obtain a "fluid" (DMPC) or "solid" (DSPC) membrane at 37°C, owing to differences in their physicochemical properties (gel-to-liquid-crystalline phase transition temperature (T_m; DMPC = 23°C, DSPC = 56°C). All lipids used in this study had purity ≥98% as tested by TLC for liposome preparation. As preliminary experiments indicated fast release of the drug from DRV lacking cholesterol, only DRV containing cholesterol were evaluated in this study. Cholesterol (Sigma), 20 or 40 mol%, was used to achieve different membrane characteristics that had been shown to influence encapsulation efficiency and stability of encapsulation (12–14). All water used in this study was purified using WaterPro PS HPLC/Ultrafilter Hybrid System (Labconco, Kansas City, MO) or equivalent instrumentation which provides low levels of total organic carbon and inorganic ions in pyrogen-free sterile water.

Two different mole ratios, 0.64 and 1.28, of BUP/lipid

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ABBREVIATIONS: BUP, bupivacaine; PL, phospholipid; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; DRV, dehydration-rehydration vesicles; CHOL, cholesterol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine.

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were used for liposome preparation at two different pHs (4.0 or 5.5).

For DRV preparation, DMPC or DSPC and CHOL were co-dissolved in *tertiary*-butanol (Fisher, Pittsburgh, PA) and lyophilized. The dried lipid mixture was hydrated with water at 60°C to form MLV. Small unilamellar vesicles (SUV) were prepared by high-pressure (8,000–10,000 psi) homogenization (15) using a single-step high-pressure homogenizer (Minilab 8.30H, APV Rannie, Albertslund, Denmark). SUV unimodal size distribution was confirmed to be 92 ± 21 nm (mean ± SD) by photon correlation spectroscopy (N4 Plus, Coulter, Miami, FL). BUP (2% or 4%) was added to the SUV, and the final pH was adjusted to 4.0 or 5.5. The liposome dispersion was then divided and transferred to glass bottles, frozen, and lyophilized overnight. To prepare DRV formulations, the lyophilized powder was hydrated first by water (20% of final volume), vortexing vigorously at 60°C, followed by addition of isotonic (150 mM) saline while vortexing at 60°C to achieve a final lipid concentration of 10%. Prior to characterization and injection, free drug was removed from the final liposomal formulations by 4 successive centrifugal washings (1000 × *g;* 5 minutes each) with isotonic saline at 4°C, followed by a final wash with hyperosmotic saline (580 mM) at 4°C. The hyperosmotic fifth wash improved bupivacaine to phospholipid (PL) mole ratio in the final product when compared with performing all five washes by isotonic NaCl. DRV batches having volume of 10–500 ml were prepared.

Liposome Characterization

BUP concentration in liposomes was determined by high performance liquid chromatography (HPLC) (1). Isopropanol (1000:1) was used to dissolve washed liposomes, and 25-μl aliquots were injected onto an 8 mm × 100 mm column (Radial-Pak 8NVCN, Waters, Milford, MA). A mobile phase of acetonitrile:phosphate buffer, 25 mM, pH 4.0 (75:25) was used, and absorption was measured at a wavelength of 210 nm. The retention time of BUP was approximately 4.7 min. Phospholipid concentration was determined using the modified Bartlett (16) or Stewart (17) procedures. The BUP to phospholipid ratio (BUP/PL) was then calculated. Liposomal size distribution was determined by photon correlation spectroscopy as described above (16).

Determination of Bupivacaine Heptane/Aqueous Phase and Octanol/Aqueous Phase Partition Coefficients

The distribution of BUP between organic and aqueous phases was determined. BUP base, 2.5, 25, or 100 mg, was added to 5 ml octanol or heptane. The aqueous phase (HEPES buffer 50 mM, titrated to four different pHs) was prepared in the range of pH 2.0–8.0). The organic phase (octanol or heptane) was combined with 5 ml of aqueous phase and mixed thoroughly by vortexing continuously for 1 h to ensure that all BUP was dissolved and reached equilibrium in its distribution between the two phases. After vortexing, the pH and BUP concentration of the aqueous phase were determined. The concentration of BUP in the aqueous phase was determined by HPLC as described above. The concentration of BUP in the organic phase was calculated by subtracting the BUP concentration in the aqueous phase from the initial organic phase BUP concentration. The organic phase/aqueous phase partition coefficient is the ratio of the concentrations: [BUP] organic phase / [BUP] aqueous phase, and the results were plotted against pH.

Membrane to Aqueous Phase Partition Coefficient

The liposome membrane to medium partition coefficient was determined as described elsewhere (20,20a) using a two-compartment system of liposome dispersions and aqueous phases separated by a semipermeable dialysis membrane.

In the first series of experiments DRV were prepared in the presence of either 4.5% or 2.1% BUP final concentration. Free drug was not removed and 1 ml of the DRV was placed in a dialysis bag and dialyzed against 50 ml of isotonic saline (pH 5.6; pH range 4.5–7.0). Dialysis was performed at room temperature (~21°C) under sterile conditions using SpectraPor dialysis membranes (50,000 MW cut-off, Spectrum Medical, Laguna Hills, CA). BUP concentrations in the dialysis bag and in the dialysate were assayed every 2 or 3 days for 3 weeks. Constant values for both compartments were reached 7–14 days after dialysis was initiated, indicating that equilibrium was reached. Every experiment was done in triplicate. Variations between each triplicate were lower than ± 10% of the average. At the end of the experiment, all the BUP introduced could be accounted for (45 mg for the 4.5% BUP and 21.7 mg for the 2.1% BUP).

In the second series of experiments, DRV were prepared having ~10% lipids but lacking BUP. One volume of the DRV was mixed with one volume of 4%, 2%, or 1.0% BUP to give final concentrations of 2%, 1%, or 0.5% BUP. Aliquots of 1 ml of these dispersions were placed in a dialysis bag and dialyzed against 50 ml isotonic saline. All other details are identical to those described above.

The details which describe the calculation of the liposome/medium partition coefficient are described elsewhere (20,20a).

Assessment of Analgesic Efficacy

All experiments were approved by the Institutional Animal Care and Use Committee. The research adhered to "Principals of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Male Swiss-Webster mice weighing 26 ± 3 g (mean ± SD) were used. Animals had free access to food and water, and were maintained on a 12-h dark–light cycle. Prior to testing, the hair overlying the abdomen was shaved. Analgesia was assessed using response to cutaneous electrical stimulation (20b). A current generator (model S48, Grass Instruments, Quincy, MA) coupled to a constant current unit (model PSIU6F, Grass Instruments) was used. The current was delivered to the skin surface by touching it gently with two electrodes fashioned from 25 G needles. The vocalization threshold (the current required to produce a vocalization response) was assessed prior to injection of study solutions. This was done by administering two successive stimuli (1 Hz), beginning at 1 mA and increasing in 1-mA increments to a cut-off of 15 mA. Mice that failed to vocalize at 15 mA were excluded from the study.

To determine analgesic duration, mice (n = 6–8 per group) were injected with 0.5% or 3.5% formulations of liposomal BUP. Plain BUP (0.5%), hyper-osmotic saline (580 mM), or drug-free liposomes were used as controls. Greater

concentrations of plain BUP were not used for control because, in preliminary experiments, concentrations greater than 0.5% were often lethal. For all groups, 150 μl of the study solution was injected subcutaneously using a 25 G needle in 8 mice. After injection, sensory block was assessed at 5, 15, and 30 minutes and then at 1, 1.5, 2, 3, 4.5, 6, 9, 12, 14, 16, 17, and 19 hours. Failure to vocalize in response to stimulation with threshold current was taken as analgesia. Testing was continued until two successive tests resulted in vocalization (i.e., absence of analgesia).

Determination of BUP Concentration at the Injection Site

In a separate group of mice, the amount of drug remaining at the site of injection after administration of 0.5% and 3.5% liposomal BUP, or 0.5% plain BUP was determined. Three animals were sacrificed at 0, 0.25, 0.5, 1, 2, 4, and 8 h after injections of all liposomal drug formulations and of free drug. For liposomal drug this follow-up was also done at 16, 24, 48, and 96 h after injection. After sacrifice, a 1 cm^2 circular tissue section including the entire area of injection, and extending to the peritoneum, was excised. The tissue was homogenized in 1 ml of isopropanol for 1 min, and then centrifuged at 16,000 × *g* (Eppendorf Centrifuge 5417C, Engelsdorf, Germany). The supernatant was diluted 100:1 in isopropanol, and BUP concentration was determined using HPLC. Results are expressed as mean ± SD.

Statistical Analysis

The durations of sensory block for the different formulations were compared by using survival analysis (log-rank test). To compare multiple groups, a Bonferroni correction was used. For in vivo release kinetics, nonlinear regression was used to determine the half-life ($t^1/_2$) of residence time at the site of injection.

RESULTS

Bupivacaine Organic Phase/Aqueous Phase Partition Coefficients

The results of BUP heptane/H_2O and octanol/H_2O phase partition coefficients are presented in Fig. 1. For both organic solvents, the organic/aqueous distribution ratio increased with increasing pH. The distribution ratios at all BUP concentrations tested were consistently greater in octanol (Fig. 1b) than in heptane (Fig. 1a). In all cases tested organic phase/aqueous phase partition coefficient did not exceed 100.0. Furthermore, a significant partition into the heptane phase occurred only when the pH exceeded 6.0, suggesting that below this pH almost all of the drug is charged, which is expected of a compound having a pK_a of 8.09 (21).

Effect of DRV Lipid Composition on DRV Size Distribution

DRV containing 20 and 40 mol% cholesterol were prepared using either the "fluid" DMPC or the "solid" DSPC as bilayer forming lipids at two mole ratios of drug to phospholipid (1.28 and 0.64), as described in **Methods**. DRV size distribution and drug to phospholipid (BUP/PL) mole ratio were determined after removal of nonliposome encapsulated BUP.

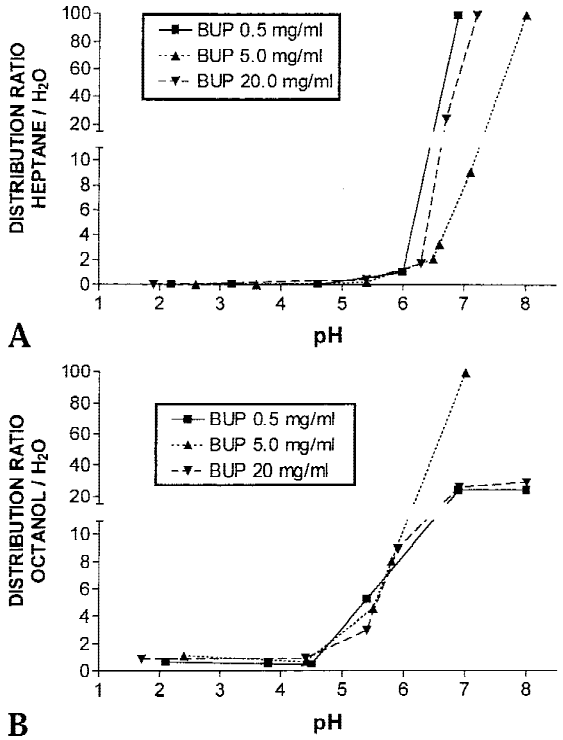

Fig. 1. Partition coefficients vs. pH for different concentrations of bupivacaine in organic solvents/water. (a) Organic solvent is heptane. (b) Organic solvent is octanol.

The results of liposome characterization are summarized in Table I. The results of size distribution measurement, based on 90° photon correlation spectroscopy, demonstrate (Table I) that the mean size of DMPC DRV was lower than that of DSPC DRV. DRV composed of 60/40 mol% DSPC/CHOL were insignificantly larger than those of 80/20 mol% DSPC/CHOL. Only for the latter composition, DRV were somewhat larger and less homogeneous at the higher (1.28) BUP/PL mole ratio.

Effect of DRV Lipid Composition on Bupivacaine/Phospholipid (BUP/PL) Mole Ratio

In general, liposomes prepared with DSPC as the bilayer forming lipid resulted in 4–9-fold-higher BUP/PL ratios. For DSPC liposomes, lower CHOL mol% resulted in higher BUP/PL ratio, whereas for DMPC liposomes, lower CHOL mol% resulted in the lowest BUP/PL ratio, and therefore this composition was excluded from further evaluation. Alteration of BUP concentration affected the BUP/PL ratio only for DSPC liposomes prepared with 20 mol% CHOL. In that case, the greater BUP concentration resulted in greater BUP/PL ratios. The highest BUP/PL mole ratio was obtained for DSPC/CHOL, 80:20 mole ratio at 1.28 BUP/PL (mole/mole). At pH 5.5 BUP solubility was 33.5 mg/ml. At pH >6.0, BUP solubility was very low. Increasing the pH to 7.0 reduced solubility 100-fold, and at pH 8.5 BUP solubility approached 0. Thus, in order to achieve sufficient loading, DRV have to be prepared at pH <6.0. However, at pH ≤5.0 the acyl ester

Table I. Liposome Characterization

#	Bilayer lipid	Cholesterol (mol%)	Added BUP (drug/lipid mole/mole)	Final BUP (mg/ml)	BUP/PL ratio[a] (mole/mole)	Size (unimodal) μm, mean ± SD
1	DSPC	40	1.28	17	0.12	2.1 ± 0.9
2	DSPC	40	0.64	19	0.13	2.1 ± 0.8
3	DSPC	20	1.28	35	0.36	1.9 ± 0.7
4	DSPC	20	0.64	29	0.26	1.7 ± 0.5
5	DMPC	40	1.28	4	0.024	1.2 ± 0.5
6	DMPC	40	0.64	5	0.023	1.3 ± 0.6

[a] Table I describes a complete comparative screening experiment. In the other experiments, in which only part of the formulations were compared, formulation 3 also has the highest BUP/PL mole ratio (>0.3).

band of the phosolipids may be hydrolyzed at a significant rate. Therefore, in order to improve chemical stability of the phospholipids (11,13) all studies for BUP DRV preparation were performed using 150 mM NaCl at pH 5.5.

Bupivacaine Liposome/Buffer Partition Coefficient

Tables IIa and IIb show the distribution of BUP between the DRV composed of DSPC/CHOL, 80:20 mol% and the extraliposomal buffer. For this we used equilibrium dialysis under two sets of conditions:

a. Starting with DRV loaded with BUP (Table IIa).
b. Adding BUP to empty DRV (Table IIb).

The experimental conditions of both sets were similar. In both, all BUP was accounted for after equilibrium was reached. When starting with BUP-DRV (set a) the liposome to buffer K_p was independent of drug concentration (comparing K_p = 14.1 and 13.1 for 4.5% and 2.1% BUP, respectively, used for drug loading of the DRV). When the equilibrium dialysis experiment was done with empty liposomes to which drug was added externally (set b), the liposome to buffer K_p was slightly lower and dependent on the drug/lipid input ratio in a reciprocal way (K_p values of 5.3, 10.4, and 16.11 were obtained for BUP concentrations of 2%, 1%, and 0.5%, respectively).

Analgesic Efficacy in Mouse Model

The formulation which yielded the greatest BUP/PL ratio (DSPC/CHOL, 80:20 (mole/mole) to which BUP at a drug/phospholipid mole ratio of 1.28 was added during DRV formation; formulation #3–see Table I) was evaluated in vivo to determine analgesic efficacy. Results for duration of sensory block are presented in Fig. 2. All mice injected with 0.5% plain BUP had analgesia at 30 min, and by 2 h no analgesia was detected. In mice given 0.5% liposomal BUP, all animals demonstrated analgesia at 3 h, and by 6 h it was not detectable in any animal. The 3.5% liposomal BUP formulation produced sensory block for 14 h in all animals, and it did not regress to baseline in all animals until 19 h. There was a statistically significant difference ($p < 0.05$) in duration of analgesia between the plain BUP and 3.5% liposomal BUP formulations and between the 0.5% and 3.5% liposomal BUP formulations, but not between the duration of the plain BUP and the 0.5% liposomal BUP formulation. No analgesia was seen after injection of saline or empty liposomes. No deaths and no apparent signs of systemic BUP toxicity (tremors or convulsions) were observed in any mice.

Kinetics of BUP Release after Subcutaneous Injection

Results of BUP release after subcutaneous injection are presented in Fig. 3. Clearance of BUP from injection site after injection of BUP-DRV shows parallel and very similar patterns for 0.5% and 3.5% BUP, except difference in "$t_{1/2}$" (3.9 h for 0.5% BUP-DRV and 8.9 h for 3.5% BUP-DRV (Table 3). As expected, the area under the curve of the 3.5% BUP-DRV was approximately one order of magnitude higher than that of the 0.5 BUP-DRV. Plain BUP (0.5%) clearance was much faster ($t_{1/2}$ = 0.14 h = 8 min) and yielded a much smaller area under the curve. The very different pharmacokinetics of plain BUP and DRV-BUP can be demonstrated from BUP concentration at the site 4 h post-injection. Only 1% of the injected dose of plain BUP remained at the site (8/750 μg), whereas approximately 54% and 66% of the 0.5% and 3.5% liposomal formulations remained (406/750 μg and 3483/5250 μg, respectively).

Table IIa. Determination of Bupivacaine Liposome Membrane/medium Partition Coefficient by Equilibrium Dialysis of BUP-DRV Against Saline

BUP concentration in DRV dispersion	Volume of DRV dispersion	Volume of dialysate	BUP inside bag (mg/ml)	BUP in dialysate	Lipid concentration (mg/ml)	BUP liposome per 1 ml dispersion	BUP/PL mole/mole	K_p (liposome/medium)	Total BUP at the end of experiment
4.5%	1 ml	50 ml	2.03	0.86	87.5	1.17 mg	0.046	15.1	45.03
2.1%	1 ml	50 ml	0.853	0.41	74.45	0.413	0.021	14.5	21.32

Note: K_p was calculated as $[BUP]_{lip}/[BUP]_{med}$. For more details see Methods and ref. 20. DRV composed of DSPC:cholesterol 80:20 mol% were used. The results are means of 2 experiments. The difference in K_p between the two experiments is 8% for 4.5% BUP and 6% for 2.1% BUP.

Table IIb. Determination of Bupivacaine Liposome Membrane/medium Partition Coefficient by Equilibrium Dialysis of Empty DRV + BUP Against Saline Containing Bupivacaine

1 ml "Empty" DRV mg/ml lipids	BUP added to DRV (mg/ml)	Dialysate volume (ml)	BUP inside bag (mg/ml)	BUP dialysate (mg/ml)	$[BUP]_{Lip}$ in 1 ml DRV dispersion	BUP/PL (mole/mole)	K_p (liposome/ medium	Total BUP at the end of experiment (mg)
43.4	20	50	0.513	0.41	0.1	0.008	5.54	20.6
39.4	10	50	0.3	0.21	0.09	0.0079	10.88	10.6
37.0	5.0	50	0.17	0.105	0.065	0.0061	16.73	5.3

Note: K_p was calculated as $[BUP]_{lip}/[BUP]_{med}$. For more details see Methods and ref. 20 and 20a. DRV composed of DSPC:cholesterol 80:20 mol% were used.

DISCUSSION

Formulation Design

Local anesthetics such as bupivacaine when administered as plain drug suffer from major drawbacks of potential systemic toxicity if a large mass of drug were to gain access to the circulation, and relatively short duration of action. Both drawbacks are related to the fast clearance of the drug from the injection site. Formulating local anesthetics in liposomal dosage forms may overcome these two major drawbacks. Additional advantages of liposome-based formulations are biocompatibility, biodegradability, and ease of injection (12–14). However, most of the many liposomal formulations of local anesthetics that have been previously tried (1,9) have a relatively low drug to lipid ratio. Therefore, their application requires injection of a large amount of lipid and relatively large volumes which make their clinical use unrealistic. This study was aimed to develop liposomes with a significantly higher than previously described (1,9) BUP to lipid ratio, which would be stable during storage, and, most importantly, drug containing liposomes would remain at the site of injection long enough while yielding controlled drug release at a level needed to achieve sufficient prolongation of analgesia.

Optimizing Drug to Phospholipid Mole (Drug/PL) Ratio

The first step in optimizing drug/PL mole ratio in passively loaded liposomes is to measure the distribution of the drug between the liposome membrane and the medium. Drugs having high (>10^6) partition coefficient (K_p(mem/med)) are defined as membrane-associated (12,14,23). When the K_p(mem/med) is low (<100), drug/PL mole ratio will be determined by drug solubility in the medium and by the liposome trapped volume.

In this study we found that bupivacaine has a low (<20.0) K_p(mem/med) (Tables IIa, IIb) as well as a low (<100) partition coefficient between media of low dielectric constant (heptane and octanol) and an aqueous phase at a broad pH range (2–8.5) (Figs. 1a,1b). Thus, the strategy to maximize the BUP/PL mole ratio should be to improve drug encapsulation in the intraliposomal aqueous phase. This was achieved by combining optimization of drug solubility and liposome trapped volume. Drug solubility was optimized by using a pH of 5.5, at which phospholipid hydrolysis during processing and storage is insignificant, and drug solubility is high. Maximization of trapped volume was achieved through the use of a dehydration-rehydration methodology. DRV-MLV, in addition to their large trapped volume, have been prepared without using organic solvents or detergents (12). Under the conditions used in this study the DRV-MLV trapped volume (measured by encapsulation of ^{3}H-inulin; ref. 15) is 3.2 ± 0.2 μl/μmole phospholipid, which is much higher than that of classical MLV (12–14).

Based on this trapped volume, when bupivacaine is at a concentration close to its solubility limit at pH 5.5 and a PL concentration of 100 mM is used for drug encapsulation, the expected BUP/PL mole ratio should be 0.39, in very good agreement with the experimental value of 0.36 ratio obtained for the optimal DSPC/Chol 80:20 MLV-DRV. For comparison, under the same conditions the amount of drug associated with the liposome membrane should result in a BUP/PL mole

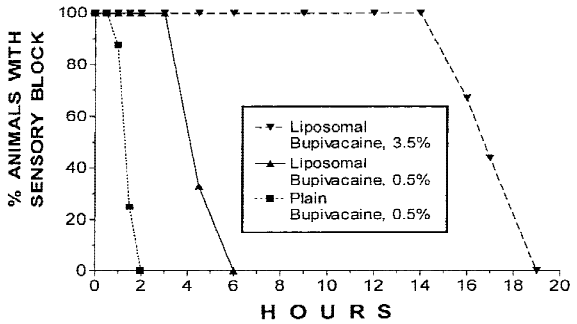

Fig. 2. Duration of sensory block of the skin overlying the abdomen after subcutaneous injection of 150 μl of 0.5% plain bupivacaine, 0.5% liposomal bupivacaine, or 3.5% liposomal bupivacaine (n = 6–8/group). The 3.5% liposomal formulation produced significantly prolonged analgesia compared to both the 0.5% plain and 0.5% liposomal formulations ($p < 0.01$).

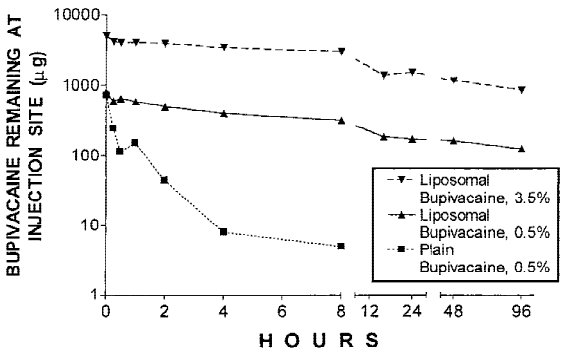

Fig. 3. Amount of bupivacaine (μg) remaining at the site of injection (subcutaneous abdominal) after administration of 150 μl of 0.5% plain bupivacaine, 0.5% liposomal bupivacaine, or 3.5% liposomal bupivacaine.

Table III. Summary of Efficacy and Kinetic Data After Subcutaneous Injection of 0.5% Plain Bupivacaine (BUP), 0.5% Liposomal Bupivacaine, or 3.5% Liposomal Bupivacaine in Mice

Formulation type	Duration of analgesia in all animals (hours)	Ratio of analgesic duration: liposomal/plain	Amount of BUP remaining at injection site at time of sensory block in all animals (μg)	Ratio of amount of BUP remaining at site: liposomal/plain	$t_{1/2}$ (hours)
Plain 0.5%	0.5	—	114	—	0.14
Lip. 0.5%	3	6	451	4	3.9
Lip. 3.5%	14	28	1550	14	8.9

ratio <0.05, which is less than one seventh of the actual value. This indicates that, indeed, most of the drug associated with the liposome resides in the intraliposomal aqueous phase. Preliminary experiments demonstrated that, as expected (12), DRV-MLV have significantly higher BUP/PL ratios than MLV prepared by conventional thin lipid hydration (data not shown).

Another advantage of DRV-MLV is their large size (~2.0 μm, Table I), which is a prerequisite for slow clearance from the injection site as demonstrated by Table 3 and Fig. 3.

Leakage of the drug from the liposomes during storage and handling is one of the major obstacles for obtaining a viable formulation (12,14). For bupivacaine, being an amphipathic weak base with a measurable (though low) partition to liposome membrane and to low dielectric organic solvents (Tables IIa,b and Figs. 1a,b), prevention of significant leakage is a difficult task. Therefore special efforts were made to minimize drug leakage by optimizing medium and membrane lipid compositions.

Medium

Special efforts were made to reduce the low dielectric organic solvent to aqueous phase partition coefficient which is a good measure of simple diffusion across lipid bilayers (26).The low medium pH of 5.5 which was used in order to improve drug solubility in the aqeuous phase also reduced heptane-to-aqueous phase partition coefficient to ~1.0 (from 100 at pH 7.5–8.0, Fig. 1a). Thereby the level of bupivacaine leakage is dramatically reduced, although at 37°C it will still be sufficient to induce analgesia.

Liposome Lipid Composition

Two parameters, well-established as major factors in controlling permeability, were studied—both related to structural discontinuities and membrane defects in the bilayer in which the small permeating molecules can reside. At the molecular level these are the kinks formed due to the formation of trans-gauche isomerizations which "run" along the hydrocarbon chain. At the organizational level there are membrane defects formed at phase boundaries. The use of long, saturated acyl chains and the presence of an optimal level of cholesterol in the lipid bilayer reduce both types of membrane "defects" (22,25) and, in parallel, lower membrane permeability (12,13,14,22). This explains why the "solid" at 37°C DSPC-based (T_m ~ 56°C) formulations had a much higher BUP/PL ratio (5–15-fold), (depending on CHOL mol%) than the fluid (at 37°C) DMPC-based (T_m = 24°C) formulations (Table 2).

Altering CHOL concentration significantly affected BUP encapsulation for both DSPC and DMPC, but in opposite directions. For DSPC liposomes, the lower CHOL concentration resulted in a greater BUP/PL ratio.

This is explained by the difference in the phase diagrams of DMPC/CHOL and DSPC/CHOL. At 37°C, DMPC-rich phases are fluid (liquid disordered = LD) and DSPC- rich phases are solid (solid ordered = SO), while CHOL-rich domains in both PCs form a new liquid ordered (LO) phase. While LO and SO phases are similar in their properties, LD and LO phases differ from one another, and therefore phase separation between LD and LO phases in the DMPC/CHOL bilayers results in more pronounced leakage (25).

Efficacy

The optimal DRV liposomes significantly prolonged duration of sensory block. Liposomal encapsulation of 0.5% BUP resulted in a 6-fold prolongation of analgesia compared to 0.5% plain BUP (Fig. 2). This prolongation of sensory block was even more profound for the higher concentration of liposomal BUP (3.5%) and resulted in a nearly 30-fold prolongation compared to the plain drug (Table 3). Furthermore, no obvious signs of toxicity were apparent at the 3.5% dose, whereas preliminary studies of plain BUP at doses greater than 0.5% produced systemic toxic effects including death. This is consistent with previous data that demonstrate that the LD_{50} of BUP is significantly increased by liposomal encapsulation (18).

Studies of BUP clearance from the injection site elucidate the mechanism of prolonged sensory block. Whereas the plain BUP was cleared from the injection site rapidly ($t_{½}$ = 8 min), liposomal formulations resulted in significantly prolonged residence of drug at the site ($t_{½}$ = 4–9 h). The kinetic studies clearly demonstrated that the duration of BUP residence greatly outlasted the duration of sensory block. This was most likely due to a release rate that became too slow to result in sufficient drug availability to produce sensory block.

A summary of the analgesic efficacy studies and local kinetics is presented in Table 3. For 0.5% plain BUP, sensory block was present in all animals for 30 min, whereas for 0.5% liposomal BUP all animals had sensory block for 3 h, and for 3.5% liposomal BUP sensory block was seen in all animals for 14 h. The relative duration of analgesia (defined as the time when all animals had sensory block) is presented as the ratio of liposomal BUP/plain BUP for both liposomal formulations (based on Fig. 2). Data are also presented for the amount of BUP remaining at the site of injection (in μg), and the ratio of remaining liposomal BUP/plain BUP at the last time point

at which sensory block was present in all animals. Finally, the time at which 50% of the injected BUP was present at the injection site ($t_{½}$) for each formulation is presented.

Some inferences on the amount of bupivacaine at the site needed to produce sensory block can be made based on analysis of the kinetic data (Fig. 3 and Table 3). Thirty minutes after injection of 0.5% plain BUP, when all animals exhibited analgesia, 114 μg was recovered from the injection site. At 2 h, when no animal demonstrated analgesia, 44 μg was recovered from the injection site. Thus, in the model used, the amount of BUP needed to produce sensory block was between 44 and 114 μg. Interestingly, for all 3 formulations, the difference in recovered BUP between the time point at which all animals were analgesic and the time point at which no animal exhibited analgesia was 70–84 μg. This seems to be the critical amount of free BUP necessary to produce analgesia as assessed by this model.

For both liposomal formulations (0.5 and 3.5% drug) throughout all the time evaluated, the level of drug in the injection site was much higher than when animals were injected with 0.5% plain drug (Fig. 3). For both liposomal treatments, when analgesia was stopped the level of bupivacaine in the injection site was much higher than the 70–84 μg needed to produce analgesia (370 μg for the 0.5% liposomal bupivacaine at 6 hours, and 1480 μg for 3.5% liposomal bupivacaine at 19 h). Namely, at these time points the level of free (nonliposomal) drug was below the threshold (44–114 μg) needed to get analgesia and most of drug measured at the site was liposome-associated and not pharmacologically available. This indicates that the DRV serve as a slow release device for bupivacaine in which most of the drug at all time points remains liposome-associated, which explains the lower level of drug in the plasma (compared with plain drug) as well as the better tolerability and lower toxicity of the liposomal drug (1,4,18).

In summary, we found that preparation of DRV-MLV loaded with BUP composed of DSPC:Chol 80:20 (mole ratio) resulted in liposomes with a favorable (0.36) BUP/PL ratio which is greater than BUP/PL ratios previously reported by others (3,19).

Furthermore, we found that this DRV-MLV BUP formulation significantly prolonged the duration of sensory block in a mouse model when compared to plain BUP.

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