

A Novel Liposomal Bupivacaine Formulation to Produce Ultralong-Acting Analgesia

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Background: Currently available local anesthetics have relatively brief durations of action. An ultralong-acting local anesthetic would benefit patients with acute and chronic pain. The authors prepared and characterized a novel liposomal bupivacaine formulation using remote loading of bupivacaine along an ammonium sulfate gradient and assessed its efficacy in humans.

Methods: A large multivesicular liposomal bupivacaine formulation was prepared by subjecting small unilamellar vesicles to successive freeze-and-thaw cycles. Bupivacaine hydrochloride was then remotely loaded into the liposomes along an ammonium sulfate gradient ($[(\text{NH}_4)_2\text{SO}_4]_{\text{intraliposome}}/[(\text{NH}_4)_2\text{SO}_4]_{\text{medium}} > 1,000$). The liposomes were then characterized for size distribution; drug-to-phospholipid ratio; *in vitro* release profile at 4°, 21°, and 37°C; sterility; and pyrogenicity. Six subjects each received six intradermal injections in the lower back with 0.5 ml of 0.5, 1.0, and 2% liposomal bupivacaine; 0.5% standard bupivacaine; saline; and “empty” liposomes. Duration of analgesia was assessed using pinprick testing of the skin directly over the injection sites. Results were compared using the log-rank test.

Results: The mean large multivesicular vesicle size was $2,439 \pm 544$ nm, with a drug-to-phospholipid ratio of 1.8, fivefold greater than results previously reported. *In vitro* release was slowest at 4°C. The median duration of analgesia with 0.5% standard bupivacaine was 1 h. The median durations of analgesia after 0.5, 1.0, and 2.0% liposomal bupivacaine were 19, 38, and 48 h, respectively. Neither saline nor “empty” liposomes produced analgesia.

Conclusions: This novel liposomal formulation had a favorable drug-to-phospholipid ratio and prolonged the duration of bupivacaine analgesia in a dose-dependent manner. If these results in healthy volunteers can be duplicated in the clinical setting, this formulation has the potential to significantly impact the management of pain.

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SUBOPTIMAL treatment of pain results in needless human suffering, and pain-related physiologic derangements are associated with medical complications, may delay hospital discharge, and may increase healthcare costs.¹ Systemic analgesics, such as opioids, produce many untoward effects, including ileus, urinary retention, somnolence, pruritus, nausea, and vomiting. The fear of respiratory depression and of inducing addiction often inhibits healthcare providers from administering sufficient drug, resulting in unrelieved pain.

Unlike systemic analgesics, local anesthetics reliably relieve pain while avoiding systemic side effects. However, currently available local anesthetics have relatively brief durations of action, so the analgesic benefit is evanescent. An ultralong-acting local anesthetic would be of great help in managing acute and chronic pain, but no such agent is yet available for clinical use. The search for the elusive ultralong-acting local anesthetic has aptly been likened to the search for the Holy Grail.²

Local anesthetics are small molecules that are rapidly redistributed from the site of injection, limiting the duration of analgesia. One approach to prolong analgesia is to complex local anesthetics with larger carriers that remain at the injection site for a prolonged time, gradually releasing anesthetic.³ Liposomes are an ideal carrier vehicle and have been used by various investigators to prolong the duration of local anesthetic action in animals⁴⁻¹⁰ and humans.^{5,11-13}

An optimal drug-to-phospholipid ratio (D/PL) is critical for a liposomal formulation to be suitable for use in humans. A low D/PL would necessitate administration of a very large lipid load to achieve the desired analgesic effect and would preclude administration of a large lipid mass in conspicuous areas such as subcutaneous tissue. Our previous studies showed that bupivacaine partitions poorly into the liposome membrane.¹⁰ Therefore, to optimize the D/PL, bupivacaine should be loaded into the intraliposomal aqueous phase.

Local anesthetics may be loaded into the intraliposomal aqueous phase using either passive or remote techniques.¹⁴ For a passive loading method, the liposomes are formed in aqueous solution that already contains the drug to be encapsulated. For liposomal bupivacaine, the highest D/PL reported to date using passive loading was 0.36.¹⁰ Optimization is not possible using passive loading for drugs such as bupivacaine because of their low solubility,¹⁴ and remote loading would be better suited to increase the level of drug in the liposomal aqueous phase. Remote loading of liposomes is dependent on establishing a gradient across the liposomal membrane

and then adding the drug to be encapsulated into the extraliposomal medium. Bupivacaine is an amphipathic weak base and therefore a good candidate for remote loading. Remote loading of bupivacaine has also been described, using a sodium citrate pH gradient, but the D/PL was only 0.26.⁸ Remote loading using an ammonium sulfate gradient has been successfully used for loading doxorubicin into liposomes.¹⁵⁻¹⁷

The current study was designed to determine whether an ammonium sulfate gradient used with a novel large multivesicular vesicle (LMVV) would yield a liposomal bupivacaine formulation with a favorable D/PL and to assess its analgesic efficacy in humans.

Materials and Methods

Preparation of Liposomes

Large multivesicular vesicles were prepared by dissolving hydrogenated soy phosphatidylcholine (iodine value of 3.0; Lipoid, Ludwigshafen, Germany) and cholesterol (Solvay Pharmaceuticals, Veenendaal, The Netherlands) in *tert*-butanol (Fisher Scientific, Morris Plains, NJ) in a 2:1 mole ratio. The solution was lyophilized, and the dry lipid "cake" was hydrated with 250 mM $(\text{NH}_4)_2\text{SO}_4$ (J.T. Baker, Phillipsburg, NJ) at 60°C to produce large multilamellar vesicles. The large multilamellar vesicles were then homogenized at 10,000–15,000 psi (EmulsiFlex-C5; Avestin, Ottawa, Ontario, Canada) to produce small unilamellar vesicles. The small unilamellar vesicles were then subjected to 10 freeze-thaw cycles between liquid nitrogen and water at 37°C to form LMVV.

Bupivacaine HCl (Orgamol, Evionnaz, Switzerland) was remotely loaded into the preformed LMVV along an $(\text{NH}_4)_2\text{SO}_4$ gradient as previously described for doxorubicin.¹⁴ To create a transmembrane ammonium sulfate gradient, the liposomes were formed in the presence of 250 mM $(\text{NH}_4)_2\text{SO}_4$, which was then removed from the extraliposomal medium by dialysis at 4°C against a 250-fold volume of normal saline, changing the dialysate three times. This formed a gradient such that $[(\text{NH}_4)_2\text{SO}_4]_{\text{intraliposome}}/[(\text{NH}_4)_2\text{SO}_4]_{\text{medium}} > 1,000$.

Next, bupivacaine was loaded by incubating the liposomal formulations with 50 mg/ml bupivacaine at a pH range of 5.0–5.5 for 45 min at 60°C (fig. 1). Nontrapped bupivacaine was separated from the LMVV by precipitating in normal saline (1,000g for 5 min at 4°C). The LMVV pellet was then resuspended in normal saline, and the process was repeated four times. The washed LMVV was then diluted with normal saline to yield formulations of 0.5, 1.0, and 2.0% total bupivacaine concentrations. The concentration of unencapsulated bupivacaine was less than one tenth of the total for each dilution. An aliquot of LMVV was not loaded with bupivacaine and was reserved for a control ("empty" liposomes).

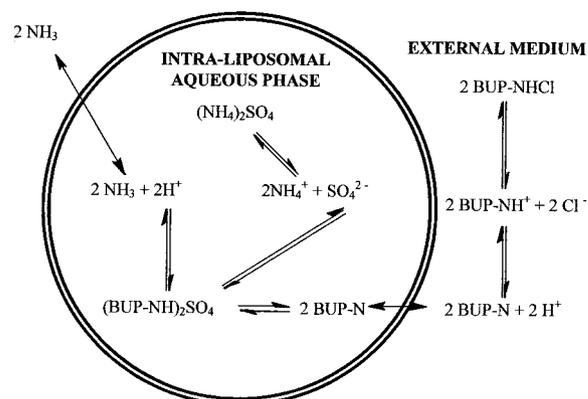


Fig. 1. Remote loading of bupivacaine into liposomes using an ammonium sulfate gradient. Note that the concentration of $(\text{NH}_4)_2\text{SO}_4$ in the liposomes is 1,000-fold greater than concentration of liposomes in the extraliposomal medium. Unionized bupivacaine (BUP) crosses the liposomal membrane and is trapped inside.

Liposome Characterization

Bupivacaine concentration in liposomes was determined by high-performance liquid chromatography.¹⁰ For this, LMVVs were solubilized with 10 volumes of isopropanol, and aliquots were injected onto an 8 × 100-mm column (Radial-Pak 8NVCN, 4 μm; Waters, Milford, MA). A mobile phase of 25 mM acetonitrile:phosphate buffer (75:25) with a pH of 4.0 was used, and absorption was measured at a wavelength of 210 nm. The retention time of bupivacaine was approximately 4.7 min. Lipid concentration of the formulation was determined using the assay of Stewart.¹⁸ The D/PL for each formulation was calculated by dividing moles bupivacaine by moles hydrogenated soy phosphatidylcholine. Liposome size distribution was determined by photon correlation spectroscopy (N4 Plus; Coulter, Miami, FL).¹⁹ To determine *in vitro* release profiles, liposomes were stored at 4°, 21°, and 37°C. At predetermined timed intervals, an aliquot was obtained, centrifuged at 1,000g to separate liposomes with encapsulated drug from free drug in the extraliposomal medium. The supernatant was assayed for the concentration of free bupivacaine using high-performance liquid chromatography. The formulations were stored at 4°C while sterility and pyrogenicity testing were performed. Sterility was confirmed by lack of growth in aerobic and anaerobic media for 2 weeks. The limulus test was used to confirm that the product was free of pyrogens (Limulus Amebocyte Lysate; Cape Cod, Inc., Falmouth, MA).

Assessment of Analgesic Efficacy

The study protocol for this first-time testing in humans was approved by the Helsinki Committee of Hadassah Hospital, Jerusalem, Israel, and the Israel Ministry of Health. Written informed consent was obtained from six healthy male volunteers. Intradermal injections were performed in the lower back of each subject with 0.5 ml

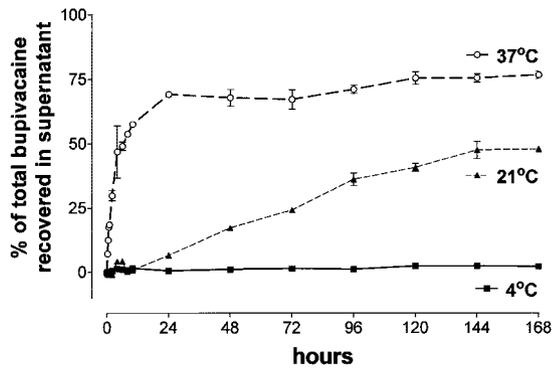


Fig. 2. Release of bupivacaine from liposomes *in vitro* at different temperature expressed as percent of total encapsulated bupivacaine *versus* time.

of six study solutions: (1) 0.5% LMVV bupivacaine, (2) 1.0% LMVV bupivacaine, (3) 2.0% LMVV bupivacaine, (4) 0.5% standard bupivacaine, (5) normal saline, and (6) "empty" LMVV (without bupivacaine). Tuberculin syringes containing the agents to be tested were coded. The investigator performing the injections outlined the border of each site and labeled it with the corresponding syringe code using indelible ink.

The lower back was chosen so that the subject would be blinded to the injected formulation. The whitish appearance of the sites injected with liposomal formulations prevented complete blinding of the assessor, who could discern that saline or standard bupivacaine had not been injected. However, the assessor did not know which of the four LMVV formulations was injected at the site (0.5, 1.0, or 2.0% bupivacaine or "empty" liposomes).

Skin sensation directly over the injection sites was assessed with a 90-mm, 26-gauge pencil-point spinal needle (Polymedic; Temena S.A., Bondy, France) at predetermined intervals after injection. To standardize the methodology, all tests were performed by holding the needle at its hub and orienting it perpendicular to the skin surface. Pressure was applied until the needle shaft bowed slightly. For each test, the subject was instructed to rate the pinprick sensation perceived over the injection sites compared to that perceived at an adjacent noninjected site, and to report the sensation as none, less, or similar. Reports of none or less were taken to indicate analgesia, while a report of similar indicated complete regression of analgesia.

Pinprick sensation was tested every hour for 15 h. Testing was suspended for 6 h to allow the subjects to sleep and was then performed at hourly intervals until 37 h, when testing was suspended again for 9 h, for sleep. Testing was then resumed at 2-h intervals. For each injection site, testing was continued only until the subject reported the perceived sensation as similar for both injected and adjacent noninjected sites for two successive intervals. The log-rank test was used to compare the analgesic duration among the study solutions.

To assess tolerability of the test formulations, the sub-

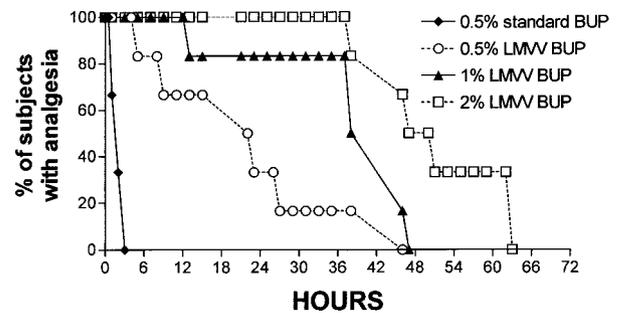


Fig. 3. Duration of skin analgesia as assessed by pinprick after subcutaneous injection of 0.5% standard bupivacaine (BUP) or 0.5, 1.0, or 2.0% formulations of large multivesicular vesicle (LMVV) liposomal bupivacaine.

jects were asked to report any discomfort that may have occurred at the injection sites for 4 weeks after administration. A physician examined the injection sites on days 1, 2, 3, 4, 7, and 28.

Results

The mean size of the LMVVs was $2,439 \pm 544$ nm. The LMVV D/PL was 1.8. *In vitro* experiments showed the bupivacaine release from LMVV was temperature dependent, occurring at a greater rate with increasing temperature, as shown in figure 2. Sterility and pyrogenicity testing confirmed that the product was sterile and pyrogen-free.

In volunteers, log-rank testing revealed that all LMVV formulations were significantly different from standard bupivacaine ($P < 0.05$). Furthermore, the prolonged analgesia produced by LMVV bupivacaine was dose related. Figure 3 illustrates the time course of the percent of subjects reporting analgesia. The median duration of analgesia after 0.5% standard bupivacaine was 1 h. The median durations of analgesia after 0.5, 1.0, and 2.0% liposomal bupivacaine were 19, 38, and 48 h, respectively.

All subjects tolerated the injections well and did not report any side effects. There was no erythema noted by the physician. However, in some subjects, the physician assessor was able to distinguish by palpation sites that were injected with liposomal preparations (irrespective of whether they were 2% LMVV, 1% LMVV, or "empty" liposomes), because they were slightly raised. In these subjects, this effect decreased with time, so that by day 28, there was no residual tenderness at the site or any other discernable evidence that an injection had been performed.

Discussion

We have described here a novel liposomal bupivacaine formulation that produced dose-dependent prolongation of local analgesia in human volunteers. Liposomes are lipid vesicles enclosing aqueous compartments into which a drug can be loaded and are ideally suited to

function as carrier vehicles, being biocompatible, biodegradable, and nonimmunogenic. Although no liposomal local anesthetic is yet available for clinical use, liposomal formulations of other pharmaceuticals, including antifungals and antineoplastics, are already marketed worldwide.¹⁹ Various liposomal local anesthetic formulations have been described by us^{6,9,10} and others.^{4,5,7,8,11} However, all of the previously described liposomal local anesthetic formulations have a relatively low D/PL, with the highest reported to date being 0.36.¹⁰ The novel LMVV formulation we used in this study had a D/PL of 1.8, an improvement of greater than or equal to fivefold over previously described formulations (0.10,²⁰ 0.26,⁸ 0.36¹⁰).

The D/PL is an important characteristic of any liposomal local anesthetic. Formulations with a low D/PL are impractical for clinical use because they necessitate administration of a large lipid load to achieve the desired analgesic effect. This precludes administration in conspicuous areas such as subcutaneous tissue, where a mass of lipid persisting at the site of injection would be noticeable long after the analgesic effect had dissipated. Therefore, achieving a high D/PL is of paramount importance in the development of a clinically useful liposomal bupivacaine formulation. The favorable D/PL in the formulation described here was achieved by a combination of two factors: (1) the remote loading technique, which concentrated the drug within the liposomes, and (2) the structure of the multivesicular liposomes, which possessed a large aqueous space into which the bupivacaine could be packed.

Remote loading has been used before to entrap bupivacaine into liposomes using a citrate gradient.⁸ In that study, the investigators adjusted the extraliposomal pH to 7.4, a pH at which bupivacaine HCl is poorly soluble. This may explain why a D/PL of only 0.26 was achieved. Although reducing the extraliposomal pH would improve bupivacaine solubility, it would weaken the citrate gradient and therefore compromise loading efficiency. To utilize the advantages of remote loading while maximizing extraliposomal bupivacaine concentration, we applied an ammonium sulfate gradient, a method that has been successfully used for loading doxorubicin and other amphipathic substances into liposomes.¹⁵⁻¹⁷ With this technique, extraliposomal pH is maintained at a range where bupivacaine HCl is highly soluble (pH 5.0-5.5).¹⁰

The *in vitro* characterization results indicate the ammonium sulfate-loaded LMVV formulation described here, in addition to having a greatly improved D/PL, also demonstrated a favorable *in vitro* release profile. We postulated that this would translate into a slow but sufficient *in vivo* local release. Indeed, using a mouse model,²¹ we demonstrated a dose-dependent prolongation of local analgesia with this LMVV formulation.²² We found that for 0.5, 1, and 2% LMVV bupivacaine, after subcutaneous injection, the mean durations of analgesia

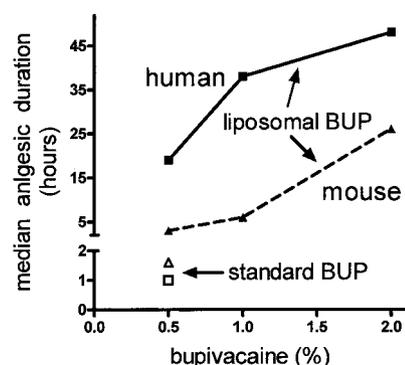


Fig. 4. Median duration of analgesia after administration of 0.5% standard bupivacaine (open triangle and open square) or 0.5, 1.0, or 2.0% liposomal bupivacaine in humans (squares) and mice (triangles; mouse data from Grant *et al.*²²).

in mice were 3, 6, and 26 h respectively, whereas 0.5% standard bupivacaine had a mean duration of 1.58 h. The human efficacy results reported here were qualitatively similar to the preclinical efficacy results in mice (fig. 4). Furthermore, the local kinetics of the formulation at the injection site in mice suggested that the explanation of the prolonged analgesia is retention of the LMVV at the site with slow but sufficient drug release.²²

In the current human study, discreet intradermal injection sites were used, and analgesia was assessed directly over each site. Results of a pilot study showed that repetitive testing with a standard hollow needle tended to puncture the epidermis. To avoid injury to the skin and iatrogenic hyperalgesia, we used a 26-gauge pencil-point spinal needle, permitting atraumatic repetitive testing. There was no potential for systemic bupivacaine toxicity in this study because each subject received a total of only 20 mg of drug.

The effective management of acute and chronic pain remains a challenge, in spite of the long-overdue recognition of this problem. Local anesthetic infiltration into wounds and around nerves innervating painful sites is a valuable but underutilized treatment option. Using local anesthetics would avoid the myriad of complications that may occur with systemic opioid administration, including respiratory depression, sedation, ileus, urinary retention, nausea, vomiting, and pruritus. However, because the duration of action of currently available local anesthetics is limited, they are not a practical option to manage pain lasting for more than a few hours. There is a compelling need for a safe and effective ultralong-acting local anesthetic.

Two approaches have been suggested to circumvent the problem of relatively limited duration of local anesthetic action. One is the continuous infusion of local anesthetic through an implanted catheter using an electronic or mechanical pump. The second approach involves encapsulation of local anesthetics within a large carrier vehicle, such as a liposome, designed to remain at the injection site and slowly release its contents.

Because slow-release local anesthetic formulations are not yet commercially available, the only option available is local anesthetic infusion. However, infusions are not without their drawbacks. The pumps are bulky, and the catheter is a potential conduit for bacteria to gain access to the tissues. Liposomal formulations, if shown to be safe and effective, would have the benefit of convenience because a single administration would provide prolonged pain relief without the need for external hardware. Furthermore, when the procedure is completed, there would be no need to continuously violate the skin barrier to administer the medication.

The drug release profile is important for the efficacy of a liposomal local anesthetic formulation. The rate of release of drug from the liposomal vehicle must be sufficient to achieve local nerve block. However, if the drug is released too rapidly, analgesic duration will not be prolonged. Furthermore, a very rapid release may result in plasma concentrations that could produce systemic toxicity. The novel formulation evaluated in this study had a favorable release profile, as demonstrated by the prolongation of analgesia.

Although the data presented with this novel LMVV formulation are very encouraging because we found that LMVV bupivacaine was well tolerated and that it significantly prolonged the duration of analgesia compared to standard bupivacaine, there are a number of issues that must be resolved before the formulation can be introduced for clinical use. Stability of the formulation during prolonged storage, batch-to-batch variability in physicochemical characteristics, and adaptability of the method for upscaling for large batch sizes remain to be determined. The primary objective of the current study was to establish proof of concept regarding the efficacy of LMVV bupivacaine in humans. The dose of LMVV bupivacaine administered in this study was low—only 17.5 mg. Before the efficacy of LMVV bupivacaine in various painful conditions can be evaluated, a study to determine its maximum tolerated dose in humans is necessary.

An ultralong-acting liposomal local anesthetic would be an ideal component of a multimodal analgesic approach. Multimodal analgesia, in which different classes of analgesics are used to inhibit distinct sites in the pain pathway, is increasingly recognized as an effective means of managing pain.²³ Moreover, multimodal analgesia permits a reduction in dose of each analgesic component, thereby decreasing the incidence and severity of side effects. By directly inhibiting nerve conduction of painful stimuli, an ultralong-acting local anesthetic would complement systemic analgesics such as nonsteroidal antiinflammatory drugs and opioids.

In summary, the present results indicate that—unlike currently used local anesthetics, which are rapidly redistributed from the site of injection—a single administration of LMVV bupivacaine provides a prolonged duration of analgesia. The novel LMVV bupivacaine formulation de-

scribed here possesses two characteristics that would favor its clinical use: A high drug-to-lipid ratio and a favorable release profile. Both are distinct advantages compared to previously described liposomal local anesthetics. The dose-related prolongation we observed suggests that the dose administered could be tailored to the patient's need by manipulating the concentration of the encapsulated drug. However, much work remains to be done before this formulation can be approved for clinical use.

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References

1. Carr DB, Goudas LC: Acute pain. *Lancet* 1999; 353:2051-8
2. Grant SA: The Holy Grail: Long-acting local anesthetics and liposomes. *Best Pract Res Clin Anesthesiol* 2002; 16:345-52
3. Grant GJ, Bansinath M: Liposomal delivery systems for local anesthetics. *Reg Anesth Pain Med* 2001; 26:61-3
4. Mashimo T, Uchida I, Pak M, Shibata A, Nishimura S, Inagaki Y, Yoshiya I: Prolongation of canine epidural anesthesia by liposome encapsulation of lidocaine. *Anesth Analg* 1992; 74:827-34
5. Planas ME, Gonzalez P, Rodriguez L, Sanchez S, Cevc G: Noninvasive percutaneous induction of topical analgesia by a new type of drug carrier, and prolongation of local pain insensitivity by anesthetic liposomes. *Anesth Analg* 1992; 75:615-21
6. Grant GJ, Vermeulen K, Langerman L, Zakowski M, Turndorf H: Prolonged analgesia with liposomal bupivacaine in a mouse model. *Reg Anesth* 1994; 19:264-9
7. Boogaerts J, Lafont N, Donnay M, Luo H, Legros FJ: Motor blockade and absence of local nerve toxicity induced by liposomal bupivacaine injected into the brachial plexus of rabbits. *Acta Anaesthesiol Belg* 1995; 46:19-24
8. Mowat JJ, Mok MJ, MacLeod BA, Madden TD: Liposomal bupivacaine: Extended duration nerve blockade using large unilamellar vesicles that exhibit a proton gradient. *ANESTHESIOLOGY* 1996; 85:635-43
9. Grant GJ, Lax J, Susser L, Zakowski M, Weissman TE, Turndorf H: Wound infiltration with liposomal bupivacaine prolongs analgesia in rats. *Acta Anaesthesiol Scand* 1997; 41:204-7
10. Grant GJ, Barenholz Y, Piskoun B, Bansinath M, Turndorf H, Bolotin E: DRV liposomal bupivacaine: preparation, characterization and in vivo evaluation in mice. *Pharm Res* 2001; 18:336-43
11. Gesztes A, Mezei M: Topical anesthesia of the skin by liposome-encapsulated tetracaine. *Anesth Analg* 1988; 67:1079-81
12. Boogaerts JG, Lafont ND, Declercq AG, Luo HC, Gravet ET, Bianchi JA, Legros FJ: Epidural administration of liposome-associated bupivacaine for the management of postsurgical pain: a first study. *J Clin Anesth* 1994; 6:315-20
13. Lafont ND, Legros FJ, Boogaerts JG: Use of liposome-associated bupivacaine in a cancer pain syndrome. *Anaesthesia* 1996; 51:578-9
14. Barenholz Y: Liposome application: problems and prospects. *Curr Opin Colloid Interface Sci* 2001; 6:66-77
15. Lasic DD, Frederik PM, Stuart MC, Barenholz Y, McIntosh TJ: Gelation of liposome interior: A novel method for drug encapsulation. *FEBS Lett* 1992; 312:255-8
16. Haran G, Cohen R, Bar LK, Barenholz Y: Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. *Biochim Biophys Acta* 1993; 1151:201-15
17. Clerc S, Barenholz Y: A quantitative model for using acridine orange as a transmembrane pH gradient probe. *Anal Biochem* 1998; 259:104-1
18. Stewart JC: Colorimetric determination of phospholipids with ammonium ferri-thiocyanate. *Anal Biochem* 1980; 104:10-4
19. Barenholz Y, Amselem S: Quality control assays in the development and clinical use of liposome-based formulations, *Liposome Technology*, 2nd edition. Vol I. Liposome Preparation and Related Techniques. Edited by Gregoriadis G. Boca Raton, CRC Press, 1993, pp 527-616
20. Legros F, Ruysschaert J: Pharmaceutical composition containing a local anesthetic and/or centrally acting analgesic encapsulated in liposomes. U.S. Patent No. 5,244,678, Ire-Celltag, S.A., 1993
21. Grant GJ, Piskoun B, Lin A, Bansinath M: An in vivo method for the quantitative evaluation of local anesthetics. *J Pharmacol Toxicol Methods* 2000; 43:69-72
22. Grant GJ, Piskoun B, Bansinath M: Analgesic duration and kinetics of liposomal bupivacaine after subcutaneous injection in mice. *Clin Exp Pharmacol Physiol* 2003; 30:966-8
23. Kehlet H, Dahl JB: The value of "multimodal" or "balanced analgesia" in postoperative pain treatment. *Anesth Analg* 1993; 77:1048-56