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Original Article

Release Pattern of Liposomal Bupivacaine in Artificial Cerebrospinal Fluid

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Abstract

Objective: We aimed to compare the possible controlled release profile of multilamellar liposomal bupivacaine formulations with non-liposomal forms in artificial cerebrospinal fluid (CSF) under *in vitro* conditions.

Methods: Liposome formulations were prepared using a dry-film hydration method. Then, an artificial CSF-buffered solution was prepared. Bupivacaine base with liposomal bupivacaine base, bupivacaine HCl with liposomal bupivacaine HCl and bupivacaine HCl were added in a Franz diffusion cell. These solutions were kept in a hot water bath for 24 h. The samples were taken at 0.5, 1, 3, 6, 12 and 24 h (1st series of experiment). Solutions of bupivacaine base with liposomal bupivacaine base and bupivacaine HCl with liposomal bupivacaine HCl were centrifuged to obtain liposomal bupivacaine base and liposomal bupivacaine HCl. Afterwards, liposomal bupivacaine base and liposomal bupivacaine HCl were added in a Franz diffusion cell. After keeping these solutions in a hot water bath for 24 h as well, the samples were taken at the same time intervals (2nd series of experiment). All samples (54 from the 1st experiment and 36 from the 2nd experiment) were analysed with high-performance liquid chromatography and ultra-performance liquid chromatography and their chromatograms were obtained.

Results: After obtaining calibration curves for bupivacaine base and HCl, release patterns of these formulations were plotted. A markedly controlled slow-release pattern was observed for multilamellar liposomal bupivacaine than for non-liposomal bupivacaine in artificial CSF.

Conclusion: Demonstration of controlled slow-release profile for mutilamellar liposomal bupivacaine in artificial CSF *in vitro* might support intrathecal use of liposomal bupivacaine *in vivo* in animal studies.

Keywords: Liposome, bupivacaine, cerebrospinal fluid, controlled slow release

Introduction

Several drugs are encapsulated into liposomes to prolong their effective duration of action (1, 2). Because liposomes can carry both lipophilic and hydrophilic drugs and deliver enzymes to cells, they may offer several opportunities for diagnosis or treatment in clinical practice. Because of the controlled release of a drug by liposomes, therapeutic effects can be achieved in low doses with the encapsulated drugs. In this way, toxic effects can be either reduced or discarded, and the half-life of a drug can be prolonged (3-6).

Local anaesthetic drugs can also be encapsulated in liposomes to prolong the duration of analgesia without increasing their toxicity. When administered via local infiltration, liposomal bupivacaine has been demonstrated to be effective for treating postoperative pain with a prolonged duration of action compared with placebo (7). In addition to its use in wound infiltration both in humans and animal models, encapsulated bupivacaine has been administered in either the intracisternal or epidural space in rabbits and subcutaneously injected in mice (7-11). However, there were no studies demonstrating the intrathecal use of liposomal bupivacaine either *in vitro* or *in vivo* until now. Therefore, in this study, we aimed to demonstrate *in vitro* controlled release pattern of multilamellar liposomal bupivacaine in artificial cerebrospinal fluid (CSF) by determining its action using high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC).

This study was presented at the 45th Annual Meeting of Society of Obstetric Anesthesia and Perinatology (SOAP) between April 24 to 28, 2013 in San Juan, Puerto Rico.
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Methods

Preparation of liposomes

Structurally multilamellar liposomes were prepared from dipalmitoylphosphatidylcholine (DPPC) and cholesterol using the dry-film hydration method as previously described by Serikawa et al. (5) and Kajiwara et al. (6) (Figures 1 and 2). Twenty mg DPPC, 20 mg cholesterol and 50 mg bupivacaine were dissolved in 6 mL methanol and dried under vacuum at 60°C. Dry film was hydrated, and liposomes were formed using a vortex mixer, followed by ultrasonication for 30 min. These formulations contained the drug both inside and outside of the liposome. Other liposome formations were separated by centrifugation. Afterwards, they were re-dispersed with 3 mL water, where the outer medium did not contain any drug molecule.

Standard solutions

- Stock standard solution of bupivacaine at a concentration of 0.1 mg mL^{-1} was prepared in a mobile phase.
- The standard solution was diluted to 1, 2, 4, 8, 16, 32 and 64 μg mL^{-1} from the stock solution.
- After removing 0.5 mL from the sample solutions of all groups, 0.5 mL CSF was added to the sample solutions of all groups and was analysed at 0.5, 1, 3, 6, 12 and 24 h.

Cerebrospinal fluid

Buffered CSF solution was artificially prepared as described in Table 1 (12). Then, CSF was used for release studies.

Release studies

First, calibration curves of bupivacaine base were determined using HPLC and UPLC. These calibration curves, as a function of concentration versus area, revealed a linear profile, with r^2=0.9999 and r^2=0.9989 for HPLC and UPLC, respectively.

All formulations were subjected to Franz-type diffusion cell experiments (Figure 3). Franz-type cells were separated by dialysis membrane, and formulations were introduced in the donor chambers where the receptor phases were CSF. An artificial CSF medium was selected to mimic *in vivo* conditions. Samples were collected from receptor compartments at pre-determined time intervals and were immediately replenished with fresh solutions.

Afterwards, release studies were performed at 37°C to consecutively analyse all samples using both HPLC and UPLC again. Chromatograms of bupivacaine base and HCl were determined using both HPLC and UPLC. These chromatograms were plotted as a function of time versus concentration.



Figure 1. Rotavapor used for preparing liposome formulations

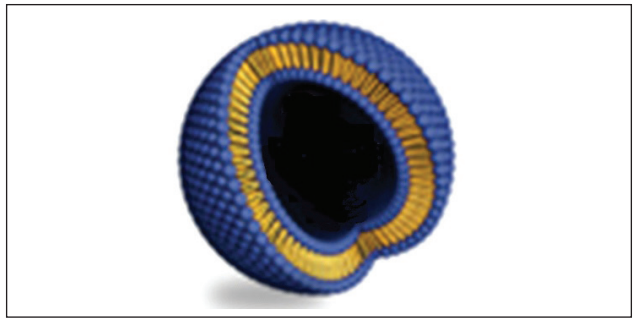

Figure 2. Cross-section of liposome where the inner space is for drug molecule

Table 1. Preparation of artificial cerebrospinal fluid

Preparation of solution A		**Preparation of solution B**	
Compound (g)		**Compound (g)**	
Dissolve in 500 mL pyrogen-free sterile water		Dissolve in 500 mL pyrogen-free sterile water	
NaCl	8.66	Na_2HPO_4-$7H_2O$	0.214
KCl	0.224	NaH_2PO_4-H_2O	0.027
$CaCl_2$-$2H_2O$	0.206		
$MgCl_2$-$6H_2O$	0.163		

Preparation of artificial CSF (combine solutions A and B in 1:1 ratio)

CSF: cerebrospinal fluid, NaCl, sodium chloride; KCl, potassium chloride; $CaCl_2$, calcium dichloride; $MgCl_2$, magnesium dichloride; Na_2HPO_4, disodium hydrogen phosphate; NaH_2PO_4, sodium dihydrogen phosphate; H_2O, water

Analytical methods (HPLC and UPLC)

- The HPLC system used was the Thermo Finnigan Surveyor (Thermo Scientific, USA) that was coupled with an ultraviolet diode array detector and automatic sampler
- The chromatographic separation was performed using a ZORBAX SB-CN column (4.6 mm × 150 m, 5 µm; Agilent, USA). The mobile phase was acetonitrile:NaH-2PO4 buffer (15 mM, pH 7.4) (50:50 v/v)
- The flow rate was 1 mL min^{-1}.
- The target compound was detected at 263 nm.

All samples (54 from the 1st experiment and 36 from the 2nd experiment) were analysed using HPLC and UPLC, respectively, and their chromatograms were obtained.

To demonstrate the release profiles of bupivacaine base and HCl forms with or without liposomes, experiments were conducted as shown below.

- Bupivacaine HCl (n=18)
- Bupivacaine HCl with liposomal bupivacaine HCl (n=18)
- Liposomal bupivacaine base (n=18)
- Liposomal bupivacaine HCl (n=18)
- Bupivacaine base with liposomal bupivacaine base (n=18)

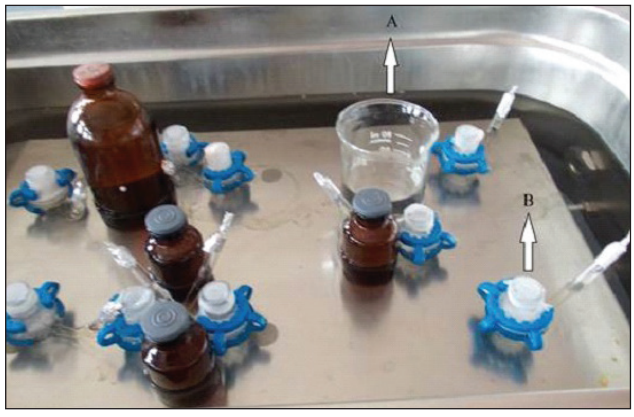

Figure 3. Franz-type diffusion cells

Results

Prior to release studies, calibration curves of bupivacaine base that were determined using HPLC and UPLC were shown in Figures 4a and 4b. These calibration curves, representing concentration versus area, revealed a linear profile, with r^2=0.9999 and r^2=0.9989 for HPLC and UPLC, respectively.

In addition, chromatograms of bupivacaine base and HCl, which were determined using HPLC and UPLC, were shown in Figures 5 and 6. These graphs were plotted as a function of time versus concentration.

Afterwards, release studies for all samples that were performed at 37°C were consecutively analysed using both HPLC and UPLC. When release profiles of bupivacaine base and HCl forms with or without liposomes were displayed, bupivacaine was found to be released from all liposomal formulations (Figure 7).

Discussion

In this *in vitro* study, bupivacaine was found to be released from all liposomal formulations. The release rates were slower depending on liposomal formulations, which might be because of the controlled release of active substance by the liposome's lipid bilayers. When liposomes were separated and re-dispersed, drug content decreased. Therefore, total released drug was found to be low; however, when the outer medium also contained the study drug, total release was found to be high. In all formulations, the lipid wall of liposome limited the drug release, which revealed that the lipid bilayer could have played an important role.

Several effects of multilamellar liposome-associated bupivacaine after epidural brachial plexus and intravenous injection in rabbits have been investigated by Boogaerts et al. (13-15). Biodistribution of liposomal bupivacaine after epidural injection was observed to be lesser than that of plain bupivacaine, possibly because of the rapid transfer of bupivacaine between the liposomes and epidural spinal nerve sheath (13). Similar to that study, we used multil-

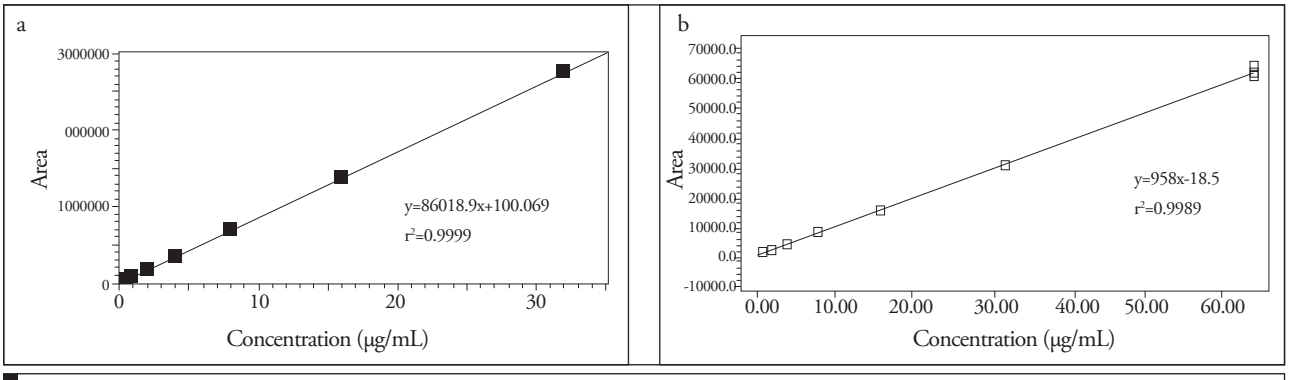

Figure 4. a, b. Calibration curves of bupivacaine base using (a) HPLC and (b) UPLC. These calibration curves, representing concentration versus area, showed a linear profile, with r^2=0.9999 and r^2=0.9989 for HPLC and UPLC, respectively

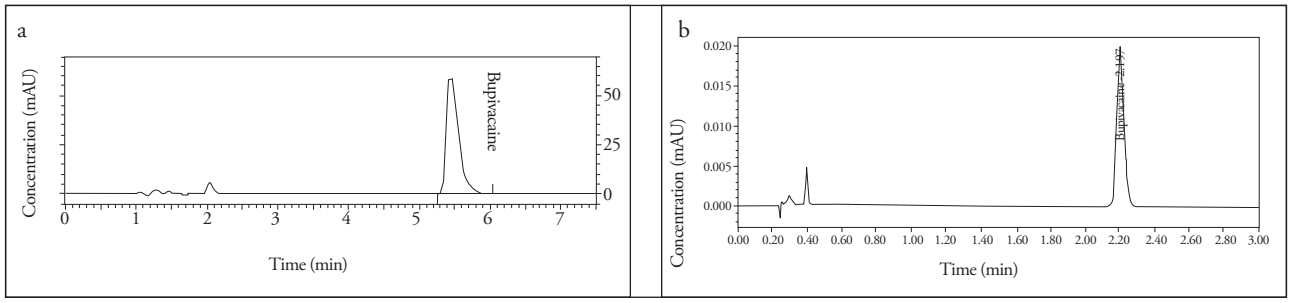

Figure 5. a, b. Chromatograms of bupivacaine base using (a) HPLC and (b) UPLC. These chromatograms were plotted as a function of time versus concentration

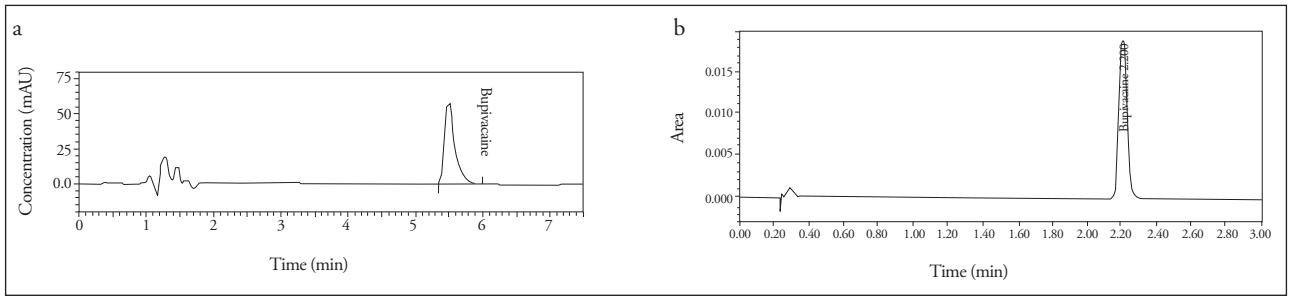

Figure 6. a, b. Chromatograms of bupivacaine HCl using (a) HPLC and (b) UPLC

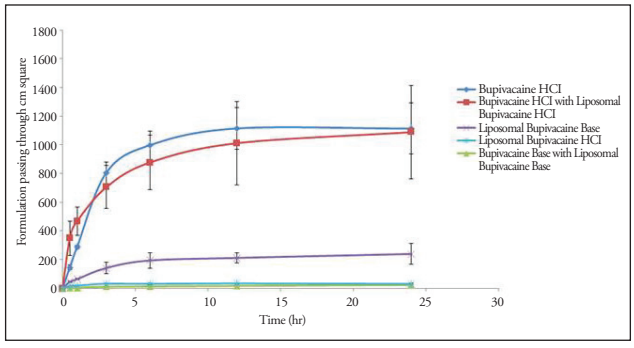

Figure 7. Release profiles of bupivacaine base and HCl forms with or without liposomes

amellar liposomal bupivacaine. However, we investigated not only two formulations, such as plain or liposome-associated form, but also compared bupivacaine base or HCL with or without liposomal formulations *in vitro*. Boogaerts et al. (13) did not demonstrate that the drug released from the liposomes could cross the subarachnoid membrane by a radiolabelled molecule. Because the release profile of the drugs could not be demonstrated due to the determination method used by them, we selected chromatographic detection (HPLC and UPLC) of bupivacaine as recommended (13). Moreover, we performed chromatographic quality controls to demonstrate that the molecular integrity of bupivacaine did not change, which was a kind of proof of the purity of the study drug.

There are several concerns with liposomal drug formulations. One of them might be the drug leakage during storage in liposomal bupivacaine formulations. Cohen et al. (16) developed a new formulation to overcome this problem. The lipid composition of liposomal bupivacaine has been modified by entrapping them into a Ca-alginate cross-lined hydrogel (bupigel). Then, significantly prolonged analgesia was achieved in mice compared with both plain and standard liposomal bupivacaine (16). Another important concern is the potential risk for neurotoxicity because of liposome-associated bupivacaine. However, after intravascular injection of plain bupivacaine with or without adrenalin at doses producing seizure, ventricular tachycardia and asystole, bupivacaine encapsulated in multilamellar liposomes reduced central nervous and cardiac system toxicities in rabbits (14).

Liposomal bupivacaine as a single dose is approved by FDA only by wound infiltration to provide prolonged postoperative analgesia (17). However, when a single epidural injection of 266 mg DepoFoam formulation was administered to healthy volunteers, prolonged duration of sensory block without extending motor block was observed (18).

Depobupivacaine (EXPAREL) is a controlled release formulation that is prepared by multivesicular technology. However, all liposomes do not have the same properties, composition and method of preparation. Multivesicular liposomes (MVL) are different from either unilamellar or multilamellar ones. MVL are larger than the traditional unilamellar (<1 μm) and multilamellar (1–5 μm) liposomes. Depofoam, which is an MVL preparation, has a non-concentric multiple lipid layer, whereas multilamellar liposomes have con-

centric lipid bilayers (19-21). This *in vitro* investigation is the first study to demonstrate the controlled slow-release pattern of structurally multilamellar liposomal bupivacaine in artificial CSF, determined using both HPLC and UPLC. Therefore, we assumed that the concentric lipid bilayer of the liposomal bupivacaine formulation might have played an important role in the controlled release pattern by limiting the drug release to a much greater extent. To the best of our knowledge, no prior study had been conducted using multilamellar liposomal bupivacaine to demonstrate the controlled release profile *in vitro* in artificial CSF. However, the primary limitation of this study is the lack of knowledge regarding the neurotoxicity potential of the liposomal drug formulation under *in vitro* conditions. If successful controlled release profiles can be achieved *in vivo* using the same liposomal drug formulations of bupivacaine, further neurotoxicity studies can be conducted to demonstrate its safety.

Conclusion

On the basis of the current *in vitro* results, intrathecal use of liposomal bupivacaine might be promising in clinical obstetric anaesthesia practice because of the slower release pattern of multilamellar liposomal bupivacaine than non-liposomal bupivacaine in artificial CSF.

Ethics Committee Approval: The present *in vitro* laboratory study was performed within the ethic rules. Since this in vitro study is demonstrating a novel approach for the drug technology development in a different media, there is no need to include any animals and human volunteers or patients requiring ethic committee approval.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - B.G., İ.T.D.; Design - A.Ü.D., İ.T.D., B.G.; Supervision - B.G., M.K.Ş.; Resources - A.Ü.D., İ.T.D., B.G.; Materials - İ.T.D., M.K.Ş.; Data Collection and/or Processing - A.Ü.D, B.G.; Analysis and/or Interpretation - İ.T.D., M.K.Ş, B.G., A.Ü.D.; Literature Search - A.Ü.D.; Writing Manuscript - A.Ü.D., B.G.; Critical Review - İ.T.D., B.G.; Other - M.K.Ş., A.Ü.D.

Conflict of Interest: No conflict of interest was declared by the authors.

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