Biodistribution of liposome-associated bupivacaine after extradural administration to rabbits

J. G. Boogaerts, N. D. Lafont, S. Carlino, E. Noel, P. Raynal, G. Goffinet and F. J. Legros

Summary

After one extradural injection of 0.25% bupivacaine 0.3 ml and 2H-bupivacaine 0.005 mCi in multilamellar liposomes, no systemic radioactivity (plasma, liver, heart muscle) was obtained for 1 h, and the labelling was less than that of systemic distribution of plain bupivacaine for the following 3 h. In contrast, radioactivity in the lumbar spinal nerves peaked in the first hour and remained higher than that of plain bupivacaine for 4 h. No radioactivity was measured in cerebrospinal fluid. Small unilamellar vesicles incorporating 3H-cholesterol did not significantly label spinal nerves and central nervous structures indicating that the mode of action of liposomal bupivacaine did not involve uptake by nerve structures. Rapid uptake of radioactivity by spinal nerves suggested exchange of bupivacaine between liposomes and nerve sheaths. (Br. J. Anaesth. 1995; 75: 319–325)

Key words


Materials and methods

Egg phosphatidylycholine (EPC T 12063–1, Lipoid K.G., Ludwigshafen, Germany) and cholesterol (Merck, Darmstadt, Germany) were the raw materials of the liposome formulations. Bupivacaine HCI was obtained from the Office Chimique (Braine-l’Alleud, Belgium). Sodium chloride, hydrochloric acid, acetic acid, sulphuric acid, sodium hypochlorite, sodium hydroxide, monosodium dihydrogenophosphate, dipotassium hydrogenophosphate, all of analytical grade, were obtained from Merck. Organic solvents were dichloromethane, chloroform, methanol and ethanol (Merck). Phosphate-buffered saline (PBS), pH8.1, 285 mosmol kg \(^{-1}\) \(\text{H}_2\text{O}\), comprised Na\(_2\)HPO\(_4\) 11.5 mg, K\(_2\)HPO\(_4\) 0.36 mg and NaCl 0.68 mg in 1 ml of water for injection.

\(\text{H}\)-Bupivacaine 1 mCi ml \(^{-1}\) (Institut des Radio-Éléments, Fleurus, Belgium) in ethanol solution was used as the marker of the local anaesthetic. The molecule was radiolabelled on the methyl group of the hydrophobic xylidine moiety. \(\text{H}\)-Cholesterol 1 mCi ml \(^{-1}\) (Amersham, Ghent, Belgium) was used as the membrane marker of the SUV bilayer. Suspensions of MLV and blood samples were spun using a bench centrifuge (Cryofuge 6–4, Heraeus Christ GMBH, Osterode, Germany). Preparation of SUV from MLV was performed by sonication using a Soniprep 150 Ultrasonic Disintegrator (MSE, Crawley, UK). Sephadex G25M (PD10 columns, Pharmacia LKB, Uppsala, Sweden) and Sepharose 4B (Pharmacia LKB) were used for separation of SUV from free bupivacaine. Radiolabelled liposomes and radioactive plasma were dissolved in a tissue solubilizer (Soluene 350, Packard, Meriden, USA). Radioactivity was counted in a liquid scintillation cocktail (Instafluor, Packard). A scintillation spectrophotometer (Rackbeta Liquid Scintillation Counter, Pharmacia LKB) was used for tritium radioactive counting.

Previous animal studies have suggested that multilamellar liposomes (MLV) remain in a depot after extradural administration [1, 2]. Bupivacaine in an MLV vehicle was associated with low, constant and prolonged plasma concentrations of drug after extradural administration [1]; Extradural administration of MLV-associated 0.5% bupivacaine to humans was associated with prolonged analgesia after surgery without motor block [3]. Extradural injection of liposomal 0.25% bupivacaine produced prolongation of pain relief without motor block in one patient suffering chronic cancer pain [4].

In this study we have examined the relationship between the clinical effects of the liposomal local anaesthetic and the extradural depot of its vehicle. Drug distribution into plasma, myocardium, liver, cerebrospinal fluid (CSF), lumbar spinal nerves and medulla was compared after injection of plain or liposomal bupivacaine into the rabbit extradural space. The local relationships of the liposomes with the lumbar spinal nerves, the medulla and brain tissues were examined by administering small unilamellar vesicles (SUV) in which 3H-cholesterol was added to the cholesterol component.

J. G. Boogaerts, MD, PhD, N. D. Lafont, MD, Department of Anaesthesiology, City Hospital, 6000 Charleroi, Belgium. S. Carlino, ENG, F. J. Legros, PhD, Department of Anaesthesiology, Flemish Free University of Brussels Medical Centre, 101 Laarbeeklaan, 1090 Brussels, Belgium. E. Noel, MD, Laboratory of Clinical Biology, Ambroise Paré Hospital, Mons, Belgium. P. Raynal, MD, Department of Surgery, CHU André Vésale, Montignies-le-Tilleul, Belgium. G. Goffinet, PhD, Laboratory of General Biology and Ultrastructural Morphology, University of Liège, Liège, Belgium. Accepted for publication: April 1, 1993.

Correspondence to F.J.L.
The size of the MLV was measured with a Coulter LS-130 particle size analyser based on laser diffraction. Electron microscopy was performed with a Jeol 100 SX transmission electron microscope.

Chemical quality controls of the MLV comprised ammonium thiocyanate (Merck), hexahydrated ferric chloride (Merck), butylhydroxytoluene (Janssen Pharmaceutica, Beerse, Belgium), glycine (Merck), thiobarbituric acid (Aldrich Chemical, Milwaukee, WI, USA), tetrahydroxypropane (Merck), lysophosphatidylcholine (Sigma, St Louis, MO, USA) and Merck 16485 silica-coated TLC plates. Bupivacaine and phospholipids were titrated using a Gilford spectrophotometer (Gilson Response Spectrometer, USA).

We used New Zealand albino rabbits, weighing approximately 2.5 kg, purchased from the Recht-persoonlijkheid Rijksstation (Ghent, Belgium). The animals were anaesthetized and sedated with Hypnorm (fluanisone and fentanyl, Janssen Pharmaceutica, Beerse, Belgium). The epicranial and 24-gauge needles were obtained from Terumo (Zellik, Belgium). Animals were killed with phenobarbitone (Nembutal, Abbott).

ASSOCIATION OF BUPIVACAINE WITH MLV

Bupivacaine was associated with liposomes made of EPC and cholesterol, as described previously [5]. Bupivacaine was dissolved in water and precipitated at pH 11 with NaOH 0.1 mol litre\(^{-1}\). Apolar bupivacaine was obtained by filtration and desiccation. Apolar bupivacaine dichloromethane solution was mixed homogeneously in a glass vial with EPC and cholesterol dissolved in the same organic solvent in a molar ratio 4 : 3. \(^3\)H-Bupivacaine in ethanol solution was added. Evaporating the solvent in nitrogen resulted in the formation of a dry lipid film made of EPC, cholesterol, lipophilic bupivacaine and its radionuclide. This film was kept overnight in a vacuum lyophilizer in order to remove the last traces of dichloromethane. PBS 1 ml, pH 8.1, was added to the lipid film, and MLV were formed by vortexing until the film disappeared completely. After 1 h at room temperature, a liposomal pellet was separated from the supernatant by centrifugation at 3500 × \(g\) for 10 min. After five successive rinsings, bupivacaine 2.5 mg and \(^3\)H-bupivacaine 0.005 mCi associated with 20 mg of lipids as MLV (BP-MLV) were suspended in PBS 1 ml. This formulation corresponded to 0.25 % bupivacaine associated with phospholipid vesicles.

PREPARATION OF \(^3\)H-CHOLESTEROL LABELLED SUV

Lipid films were made of EPC and cholesterol in a molar ratio 4 : 3, and in a total amount of 20 mg and \(^3\)H-cholesterol 0.005 mCi. After complete evaporation of the organic solvent in nitrogen, PBS 1 ml pH 8.1, was added. After vortexing, MLV suspensions were obtained. SUV were prepared by ultrasonic disintegration of MLV for 15 min at 15 MHz and 0 °C in nitrogen. The liposomal suspension was centrifuged at 3500 \(g\) for 20 min in order to discard unsonicated MLV and probe titanium particles. Suspensions of 20 mg of SUV inserting \(^3\)H-cholesterol 0.005 mCi per millilitre of buffer were thus obtained.

MORPHOLOGICAL, PHYSICAL AND CHEMICAL CHARACTERISTICS OF MLV ASSOCIATED WITH BUPIVACAINE

Quality controls of the liposomal bupivacaine formulations were performed following the recommendations of Barenholz and Amselem [6] and Betageri, Jenkins and Parsons [7] for preparations of liposomal drugs for human use. These quality controls focused on morphology, size of the liposomes associated with the local anaesthetic and chemical integrity and dose of bupivacaine and phospholipids. All quality control assays were performed on liposome-associated 0.25 % bupivacaine without radionuclide.

Phospholipid vesicles were observed by freeze-etching electron microscopy [8]. Liposomal droplets were frozen by direct immersion in liquid Freon 22 and maintained in liquid nitrogen (−196 °C). After introducing the frozen blocks into the vacuum chamber at −150 °C (Balzers BAF 400 freeze-etching unit), they were cracked with a knife blade. Sublimation of water (freeze-drying) was produced for 1 min at −100 °C. The etched faces were converted into replicates after platinum shadowing (45°, 8 s) and carbon coating. The specimens were dissolved away through H\(_2\)O, H\(_2\)SO\(_4\) and sodium hypochlorite. Replicates were viewed in a transmission electron microscope.

The sizes of the MLV were measured using a Coulter LS-130 particle size analyser based on laser diffraction of light scattered from particles exposed to a collimated light beam. Auto-rinsing of the particle size analyser was performed with PBS 3 litre and the run cycle was performed with 3-ml liposomal preparations. All operations were computerized, including statistics. This vesicle-sizing method is currently applied to liposomes [9]. Results were analysed following the recommendations of Ruf, Georgalis and Grell [10] and Hauser [8].

The chemical integrity of the bupivacaine molecule associated with MLV was determined by infrared (IR) and ultraviolet (UV) spectroscopy using the spectra provided by the manufacturer and by the pharmacopoeia as references. Bupivacaine was extracted from liposomes by acidification and then measured by UV spectrophotometry. A sample of the liposomal suspension (1.5 ml) was poured into a test tube of 5 ml and centrifuged at 3500 \(g\) for 10 min at 4 °C. The supernatant (1 ml) was diluted by 1 ml of HCl 0.2 mol litre\(^{-1}\). Absorption was read at 270.5 nm against a blank of HCl 0.1 mol litre\(^{-1}\). Bupivacaine and apolar bupivacaine in acid solution (HCl 0.1 mol litre\(^{-1}\)) were tested as controls.

A direct method based on the complex formed between the phosphate group of the phospholipids and ammonium ferrothiocyanate [11] has been used routinely for phospholipid titration. This complex was extracted into chloroform and determined spectroscopically. This method was very rapid and sensitive from 10 to 100 nmol [12]. To a sample of liposomal suspension containing not more than 50 μg
of phospholipids in buffer were successively added
cholorofrm 1 ml and 2 ml of a solution of hexa-
hydrated ferric chloride 0.1 mol litre$^{-1}$ and
ammonium thiocyanate 0.4 mol litre$^{-1}$, followed by
choleform 1 ml. This mixture was vortexed for at
least 2 min. After 5 min of centrifugation at 2000 g,
4 °C, the supernatant was discarded and the optical
density of the cholorofrm lower phase was measured
at a wavelength of 470 nm. The concentration of phospholipids was calculated in comparison with
standard solutions of EPC from 1 to 100 μg ml$^{-1}$.

Lipid peroxides were estimated as thiobarbituric
acid reactive material (TBARM) by the thiobarbituric acid test [13]. The accuracy of the method
was limited to TBARM 2 nmol [14]. To 0.1 ml of the
solution to be assayed were added 0.1 ml of hexa-
hydrated ferric chloride solution 0.01 mol litre$^{-1}$,
0.1 ml of a butylhydroxytoluene solution in ethanol
as antioxidant 0.01 mol litre$^{-1}$, 1.5 ml of glycine
buffer 0.3 mol litre$^{-1}$ (pH 3.6) and 1.5 ml of 0.5 %
thiobarbituric acid in a 0.3 % sodium dodecyl
sulphate solution in water. The mixture was vortexed
and heated for 15 min in a boiling water bath. After
cooling in an ice bath, glacial acetic acid 1 ml and
cholorofrm 2 ml were added. The tube was vortexed
for 15 s and centrifuged for 10 min at 2000 g,
4 °C. The optical density of the cholorofrm phase
was measured at a wavelength of 532 nm. The
amount of TBARM was determined in comparison
with a standard solution of tetrahydroxypropane
hydrolysed under the same conditions. Results were
reported as nmol TBARM in the phospholipid
suspension.

Semi-quantitative determination of neurotoxic
lysosphatidylcholine was carried out by thin
layer chromatography (TLC) [6]. The solution
tested was extracted by the method described by
Bligh and Dyer [15] with some modifications. To
0.1 ml of the solution to be tested were added
methanol 0.1 ml and chloroform 0.1 ml. The mixture
was vortexed for 15 s and centrifuged for 10 min at 2000 g,
4 °C. The supernatant was discarded and a
0.01-ml spot of the lower chloroform phase was
settled on the baseline of a 10-cm, silica-coated
TLC plate. The eluent system was a mixture of
chloroform–methanol–water in a volume ratio of
65/25/4. After development, the plate was revealed
by exposure to iodine vapours. Retention factors
(Rf) of EPC and lysosphatidylcholine were
determined with a standard solution in chloroform.
It appeared by chromatography of successive dilu-
tions that the lowest amount of lysosphatidyl-
choline to be detected was 5 μg ml$^{-1}$.

EXTRADURAL INJECTION TO RABBITS

New Zealand albino rabbits were anaesthetized with
Hypnorm 0.1 mg kg$^{-1}$ i.m. followed after 2 h by
0.05 mg kg$^{-1}$. The extradural space was identified by
loss of resistance to air and all solutions administered
did not exceed 0.5 ml.

One extradural injection of a 0.3-ml solution
containing 0.25 % bupivacaine 2.5 mg ml$^{-1}$ and 1H-
bupivacaine 0.005 μCi ml$^{-1}$ was performed at the
sacral level (S2–3). Bupivacaine was either in the
native hydrochloride form (pH 6.5) or associated with the liposomal preparations (MLV 20 mg ml$^{-1}$,
pH 8.1) described above. No animal died from cardiotoxic effects.

The radioactivity plasma level was determined by
counting arterial blood. A 24-gauge needle was
inserted into the ear artery. Blood (2 ml) was
withdrawn into heparinized tubes at various times
after extradural injection over the following 4 h. The
blood samples were centrifuged at 3500 g for 15 min.
Plasma samples of 0.5 ml were mixed with Soluene
1 ml in Packard glass vials, Instafluor 10 ml was
added, and beta counting was performed after 1
night at 4 °C.

The plasma level of radioactivity, expressed as a
percentage of the injected dose (% ID) in the total
plasma of the animal, was calculated as follows: (cpm
in 1 ml plasma × total cpm injected$^{-1}$) × 32.8 × 2.5 ×
100 = % ID in plasma, considering a rabbit
plasma volume of 32.8 ml kg$^{-1}$ [16]. The method was
sensitive to 0.01 % ml$^{-1}$ and was reproducible
(σd < 5 % of the mean value for 10 measurements of
the same sample) from 0.05 to 10 % ml$^{-1}$.

BIODISTRIBUTION OF PLAIN AND LIPOSOME-
ASSOCIATED BUPIVACAINE

Animals (n = 6 at each experimental time) were
killed from 15 min to 4 h by i.v. administration of
phenobarbitone 5 ml. Before killing, CSF 0.5–1 ml
was collected in suboccipital puncture at the level of
the cisterna magna. Immediately after death, the
vertebral arches were opened dorsally and lumbo-
sacral spinal nerves were dissected. Three samples
were rinsed repeatedly in NaCl 150 mmol litre$^{-1}$,
blotted arid weighed. They were dissolved for 48 h at
37 °C in Soluene 1 ml. The abdominal cavity and the
thoracic wall were incised. The liver and heart were
removed, rinsed in NaCl 150 mmol litre$^{-1}$, blotted
and weighed. Three samples of each organ weighing
approximately 1 g and CSF 0.1 ml were dissolved in
Soluene 1 ml and counted in Instafluor 10 ml. After
correction for quenching, results were expressed in
reference to the ID (% ID) incorporated in CSF
1 ml, in the whole organ (liver, heart) or in 1 g of
tissue (spinal nerves). The method was sensitive
from cpm countings corresponding to 0.02 % ID g$^{-1}$
and was reproducible (σd < 5 % of the mean value for
10 measurements of the same sample) from 0.05
to 40 % ID g$^{-1}$.

BIODISTRIBUTION OF 1H-CHESTEROL-LABELLED SUV
IN LOCAL AND MEDULLARY NEURAL TISSUES

Five animals were killed 15, 30, 60, 180 and 240 min
after extradural injection. Before sacrifice, CSF
0.5–1 ml was collected by suboccipital puncture at the
level of the cisterna magna. The spinal cord was
opened by section of the vertebral arches from the
cervical to the sacral level. The spinal cord was
dissected in thoracic and lumbar sections. The cauda
equina, the meninges and several lumbar spinal
nerves were isolated. After opening of the skull, the
brain was dissected and three samples weighing
approximately 1 g were obtained. Before beta count-
ing, CSF 0.1 ml and nerve tissues were diluted in tissue Soluene 1 ml and Instafluor 10 ml. The results were expressed as the % ID in 1 g of tissue or 1 ml of CSF.

STATISTICAL ANALYSIS
Results are expressed as mean (sd). Statistical significance was determined using the one-way ANOVA test with repeated measurements and unpaired Student’s t test for comparison between the two groups. \( P < 0.05 \) was considered statistically significant.

Results
Table 1 summarizes the physical and chemical characteristics of five different preparations of liposome-associated 0.25 % bupivacaine. The chemical integrity of the local anaesthetic molecules associated with MLV was proved by IV and IR spectrometry. The UV spectograms obtained with bupivacaine extracted from liposomes were identical to the reference spectrum found in the pharmacoepia and to the spectra from acid solutions of bupivacaine and apolar bupivacaine. Absorption occurred at 270.5 nm and the molar extinction coefficient was 338 ml mg \(^{-1}\) cm \(^{-1}\). The IR spectogram was identical to the reference spectrum of the pharmacopeia. Liposomal EPC injected into the rabbits’ extradural spaces did not present any detectable neurotoxic lysophosphatidylcholine or cytotoxic peroxides using methods recommended for human trials [6, 7]. Laser Coulter statistics showed that the polydispersity of the drug carriers was broader than that of the stannous oxinate MLV used for scintigraphic biodistribution studies [2] (table 2). An electron micrograph of MLV-associated bupivacaine confirmed the multilamellarity and, dispersity of the vesicles.

The kinetic profile of plasma radioactivity from 15 min to 4 h after extradural administration of bupivacaine and bupivacaine–MLV (BP–MLV) is shown in figure 1. Radioactivity was not observed in plasma during the first 30 min after injection of BP–MLV while it peaked at 1.65 % ID at the same

---

**Table 1** Physical and chemical quality controls of liposome-associated 0.25 % bupivacaine suspensions (BP–MLV) \((n = 5)\)

<table>
<thead>
<tr>
<th>Quality controls</th>
<th>0.25 % BP–MLV</th>
<th>MLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology (freeze-etching)</td>
<td></td>
<td>MLV</td>
</tr>
<tr>
<td>Median size population ((\mu m))</td>
<td>0.145</td>
<td></td>
</tr>
<tr>
<td>Median size volume ((\mu m))</td>
<td>5.794</td>
<td></td>
</tr>
<tr>
<td>80 % Population size limits ((\mu m))</td>
<td>0.106-0.270</td>
<td></td>
</tr>
<tr>
<td>80 % Volume size limits ((\mu m))</td>
<td>1.771-11.38</td>
<td></td>
</tr>
<tr>
<td>Liposomal BP UV spectrum</td>
<td>OK</td>
<td></td>
</tr>
<tr>
<td>Liposomal BP IR spectrum</td>
<td>OK</td>
<td></td>
</tr>
<tr>
<td>Lysophosphatidylcholine ((\mu g ml^{-1}))</td>
<td>&lt; 5</td>
<td></td>
</tr>
<tr>
<td>Peroxides (nmol)</td>
<td>&lt; 2</td>
<td></td>
</tr>
</tbody>
</table>

---

**Table 2** Cumulative distribution Coulter statistics of multilamellar vesicles associated with bupivacaine (BP–MLV) or stannous oxinate (SnOx–MLV). Size and volume of liposomes are expressed as cumulative percentages \((10, 25, 50, 75, 90)\) of the total population

<table>
<thead>
<tr>
<th>Cumulative %</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP–MLV ((\mu m))</td>
<td>0.270 0.194 0.145 0.116 0.106</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SnOx–MLV ((\mu m))</td>
<td>0.272 0.190 0.143 0.115 0.105</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP–MLV ((\mu m))</td>
<td>11.38 8.819 5.794 3.421 1.771</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SnOx–MLV ((\mu m))</td>
<td>8.326 5.626 3.455 2.089 1.231</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Figure 1  Kinetics of total plasma level radioactivity at different times after extradural injection of 0.3 ml of 0.25 % bupivacaine and \(^3\)H-bupivacaine 0.005 mCi ml \(^{-1}\), plain (BP) (●) or associated with multilamellar liposomes (BP–MLV, 20 mg of lipids per millilitre of buffer, pH 8.1) (◇). Results are expressed as a percentage of the injected dose (ID) in the whole plasma of six rabbits at each experimental time (mean, \(sd\)). \( P < 0.001 \) between BP and BP–MLV at each experimental point using Student’s \(t\) test; \( P < 0.001 \) between values at 30 and 120 min in the BP curve using ANOVA test.

Figure 2  Hepatic labelling (expressed as a percentage of the injected dose (ID) in the whole organ) at different times after extradural injection of 0.3 ml of 0.25 % bupivacaine and \(^3\)H-bupivacaine 0.005 mCi ml \(^{-1}\), plain (BP) (◇) or associated with multilamellar liposomes (BP–MLV, 20 mg of lipids per millilitre of buffer, pH 8.1) (●). Each experimental point represents the mean, \(sd\), of six rabbits. \( P < 0.05 \) between the two curves at each experimental point using Student’s \(t\) test; \( P < 0.01 \) between values at 15, 30 and 60 min and values at 240 min in the BP curve using ANOVA test. No significant difference between values at 15, 30 and 60 min of the same curve. ANOVA test also indicated that in the BP–MLV group, the value at 60 min was statistically different from those at 120 and 240 min \( (P < 0.05) \).
time as when using plain bupivacaine. The plasma concentration of drug originating from liposomes increased to 0.5% ID after 1 h and remained constant during the next 3 h (fig. 1).

Hepatic radioactivity averaged 10% ID after 30 min and increased to 20% ID 4 h after extradural injection in the BP group. There was no radioactivity in the liver in the first 30 min when using liposome-associated bupivacaine. Hepatic accumulation increased slowly to 7.5% over 2 h and did not change after 4 h (fig. 2). Similar results were obtained in myocardium.

Radioactive labelling of the lumbosacral spinal nerves averaged 1% ID/gH10021 during the first hour after injection of plain bupivacaine. During the same period, radioactive accumulation from BP–MLV averaged 15% ID/gH10021. Spinal nerves labelling decreased respectively to 0.1 and 2% ID/gH10021 (P<0.01) after 4 h in both experimental conditions (fig. 3).

Radioactivity counted in the lumbar spinal cord peaked at 7% ID g–1, 15 min after administration of plain bupivacaine. It decreased after 30–60 min, and almost no labelling was present after 2 and 4 h (fig. 4). In contrast, radioactivity increased slowly for 30 min after extradural administration of the liposomal aminoamide, remained at this level for 1 h and subsequently declined to values corresponding to those found after injection of plain local anaesthetic (fig. 4). These results demonstrate the inverse relationship between labelling in spinal nerves and lumbar medulla with two formulations of bupivacaine (table 3). There was no radioactivity in CSF 4 h after injection of liposomal bupivacaine (table 4).

Radioactivity in the brain, thoracic and lumbar spinal cord, lumbar spinal nerves, cauda equina and meninges after extradural injection of 3H-cholesterol-labelled SUV was less than the limit of sensitivity of the method.

**Discussion**

The quality controls showed that the association of bupivacaine with MLV did not alter its molecular integrity, as observed by UV and IR spectrometry. Bupivacaine was seen as crystals inserted into and outside the MLV. The range of size of the liposome drug vehicles was greater than that of stannous
oxinate MLV used for scintigraphic study [2]. This indicates that the extradural depot of liposomes may be extrapolated to MLV-associated bupivacaine.

The association of bupivacaine with MLV altered the blood and tissue distribution profiles of the drug after extradural injection. As described previously [1], the systemic concentrations (plasma, liver, myocardium) of bupivacaine were lower for 4 h compared with those after administration of an equal dose of plain drug. In contrast, the concentration of bupivacaine was 10–15 times higher in the lumbo-sacral spinal nerves for 4 h after extradural administration of BP–MLV. The labelling of brain, the thoracic and lumbar spinal cord, lumbar spinal nerves, cauda equina and meninges after extradural injection of $^3$H-cholesterol-labelled SUV was at the inferior limit of detection of radioactivity counting. The pharmacokinetic studies were conducted for 4 h because human postoperative pain relief after extradural injection of MLV-associated 0.25% bupivacaine is approximately 4–5 h [17].

There was no systemic labelling for the 30–60 min after extradural injection of liposome-associated bupivacaine and during this time spinal nerve concentrations reached a peak. Subsequently, systemic labelling increased while local nerve tissue radioactivity decreased. This behaviour may be explained by the properties of liposomes vehiculating drugs.

Phospholipid vesicles are known to release their drug content in a slow and controlled manner [7]. This may account for the delay in vascular absorption from the extradural MLV depot leading to delayed plasma, myocardial and hepatic radioactivity. The systemic toxicity of bupivacaine is associated with its plasma concentration [18, 19]. The low and constant plasma concentrations of the drug with BP–MLV suggests a reduction in systemic toxicity.

Uptake by local nerve structures may be explained in terms of the relationships of liposomes with the phospholipid bilayers of the nerve sheaths and of the nerve cell membranes. The hypothesis of fusion between the phospholipid bilayers of the liposomes and the cell membranes is now largely considered as a low level process [20]. The difficulty in obtaining uptake by local nerve structures in vivo has been noted [21]. The cell fusion hypothesis has been largely replaced by the concept of endocytosis [7, 20, 21]. This mode of action suggests that target cells are endowed with phagocytic properties. However, this is not the case for the nerve sheaths of the spinal nerves. Liposomes cannot cross the epineurium and endoneurium and this was confirmed by the absence of radioactivity from $^3$H-cholesterol-labelled SUV (diameter 0.05 μm) in all nerve structures. In addition, this absence of radioactivity allows us to discard the hypothesis of fusion of the liposomes with the nerve sheath phospholipid bilayers. The SUV used in our study are known to undergo both the greatest degree of phagocytosis and also the highest probability of cell fusion [7, 20].

It has been shown that liposomes labelled by a pyrenyle radical could transfer almost immediately their marker to the cell membrane by exchange between the two phospholipid bilayers [22]. Such an immediate transfer of bupivacaine between the liposomes and extradural spinal nerve sheaths would establish a concentration of bupivacaine inside the nerve membranes sufficient to ensure rapid analgesic effect of the drug without active accumulation at the level of the inner motor C-fibres. This would account for the rapid onset and prolonged sensory block without motor block observed in post-surgical analgesia [3].

The reasons why the drug released from the liposomes did not cross the subarachnoid membrane are not clear. The absence of bupivacaine in the CSF after administration of liposome-associated local anaesthetic should be reinvestigated using liquid or gas chromatography. This is important as it has been reported that diffusion of bupivacaine into the spinal cord via the CSF would play an important part in the motor block produced by local anaesthetics administered extradurally [23]. The lack of this phenomenon with BP–MLV may explain the absence of motor block observed in clinical trials [2].

The plasma and tissue measurements of bupivacaine were based on $^3$H-bupivacaine radioactivity counting. Tritium was chosen because it is a stable isotope with a half-life of 12 yr. Bupivacaine is catabolized rapidly into more polar compounds by the liver. The radioactivity measured in the rabbit plasma thus included bupivacaine and catabolic products and did not represent true local anaesthetic concentrations. Tritium labelling of bupivacaine was performed on a methyl group associated with the benzoyl lipophilic moiety of the molecule and hepatic biotransformation of the local anaesthetic does not change this part of the molecule. The main purpose of the present study was limited to a comparison of tissue distributions of bupivacaine using the two formulations, plain and liposome-associated. The feasibility of a stable isotope method has been demonstrated in similar experiments [24]. However, qualitative chromatographic detection of bupivacaine and known metabolites is required to clarify these issues.

Acknowledgements

We thank Mr Jean Coel for excellent technical assistance and Mr Marcel Geens, MD, for help in the preparation of the manuscript. This work was supported partly by the Ministry of Public Health of the Brussels Region and by Therapeutica SA, Brussels, Belgium.

References


Biodistribution of liposomal bupivacaine