

Research Article

**Drug Compatibility and Distribution
Assessments of a Bupivacaine-Polymer
Formulation Measured Following Site-Directed
Injection in Mice**

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A B S T R A C T

Background: A long-acting local anaesthetic agent would enable superior post-operative analgesia. Our slow-release bupivacaine-polymer formulation comprising 15% bupivacaine loaded into a poly (DL-lactic acid co castor oil 3:7), DL-LA:CO was effective for up to 96 hours. In this study we determined *in vivo* drug levels of the formulation from plasma and tissue assay and we tested the feasibility of drug level

assessments from an exudate sample collected in a novel device near the injection-site.

Methods: Prior to formulation injection, a titanium cylinder dual-open-ended chamber was implanted in the dorso-lumber region of female ICR mice, to be used as a biological compartment. Following a healing period, the bupivacaine-polymer formulation was injected close to the sciatic nerve. Formulation assay samples were collected over one-week post-injection; exudate was drawn from the chamber in the anaesthetised animal; and blood and tissue samples from the sacrificed animal. Bupivacaine distribution in the exudate and plasma samples was determined by liquid chromatography–mass spectrometry developed for these experiments, and in tissue samples by high -performance liquid chromatography.

Results: Bupivacaine was detected during the first 24h following formulation injection in all three samples (exudate, plasma and tissue), and decreased to almost undetectable levels by day 7 post-injection.

Conclusion: Bupivacaine drug assay measured from an exudate sample may be applicable in addition or as a replacement for standard plasma and tissue assay in studies of drug pharmacokinetic distribution. The high sensitivity and simplicity of the metallic chamber implant to detect drug concentration and exudate analysis was confirmed in sever hepatic impairment.

Keywords: Biodegradable polymer, Bupivacaine, Castor oil, Lactic acid, Pharmacokinetics.

Abbreviations: ACN: Acetonitrile; CXP: Cell Exit Potentials; HPLC: High-Performance Liquid Chromatography; DL-LA:CO: Poly(DL-Lactic Acid Co-Castor Oil 3:7); DDW: Double Distilled Water; ESI: Electrospray Ionisation; IPA: Isopropanol; MRM: Multiple Reaction Monitoring; MP: Mobile Phase; MS: Mass Spectrometry; LOD: Limit of Detection; LOQ: Limit of Quantification; IS: Internal Standard; LC/MS: Liquid Chromatography–Mass Spectrometry

INTRODUCTION

Long-acting local anesthetic agents can provide superior analgesia for pain management is important, since efficacy of

these drugs in a clinical setting is limited by short duration of action¹. Long-acting site-directed local anesthetics can prolong the duration of pain relief; usually achieved

through continuous catheter drug infusion. Recent advances have led to clinical availability of long-acting local anesthetic agents for directly placed site-directed application^{2,3}.

Alternative options for site-directed long-acting local anesthetic agents are in development. We previously reported up to 96 hours of analgesia following site-directed injection application of a slow release of a poly (DL-lactic acid co castor oil 3:7), DL-LA:CO, formulation loaded with 15% bupivacaine, a local anesthetic agent⁴.

Assessment of local toxic effects is an essential step during drug development⁵. The standard measurement of toxic drug effects is through plasma and tissue sampling that involves sacrificing the animal to obtain the plasma and tissue samples. Such drug assays can be performed in the supernatant by High-performance liquid chromatography (HPLC); an accurate and rapid method for the measurements required for regulatory approval⁶. Bupivacaine concentration in human plasma has been determined using HPLC⁷⁻¹⁰ and mass spectrometry (MS)^{6,11}.

In the current work, we developed a novel method of *in vivo* tissue sampling through an exudate sample obtained from the injection site, in addition to samples obtained for bupivacaine drug assays using standard measurement from tissue and plasma, in order to determine drug concentration and compare the drug distribution in these three samples after injection of the bupivacaine-polymer formulation.

MATERIALS AND METHODS

The study received approval from the Ethics Committee of the Hebrew University Hadassah Medical School (OPRR-A01-5011) for performance of animal studies (MD-11-12672-4). Experiments were performed following the ARRIVE guidelines. Female ICR mice (25 g-35 g) were housed in the Specific Pathogen Free laboratories of the Hebrew University, ten per cage with free access to food and water. The animal room was light cycled (12 h light, 12 h dark), and the temperature was 22°C.

The exudate samples

A subcutaneous inert titanium chamber (1.5 cm) was used as a biological compartment, and its exudate content sampled at specified time intervals without need to sacrifice the

animals. Titanium is highly biocompatibility thus local inflammatory reactions were avoided (Figure 1). This selected model was developed to examine, the host response and the effect of anti-inflammatory drugs on the immune response^{12,13}. The titanium chamber was implanted in the subcutaneous tissues in the left dorso-lumbar region of the mice following anaesthesia with an intraperitoneal injection of ketamine: xylazine (80: 20 mg/Kg)^{12,13}. The chamber was easily palpated under the skin and permitted easy needle access to withdraw the exudate that gathered as it was open from both sides^{12,14}. The first week following chamber implantation was a healing period, as the chamber became covered by loose connective tissue, rich in blood vessels. During this healing period, animals were weighed and followed daily for behavioural changes.

Formulation raw materials

DL-lactide Purasorb, Purac Amsterdam; Tin(II) 2-ethylhexanoate, (Sn(Oct)₂), Sigma Israel; castor oil, Tamar Pharm Israel; bupivacaine HCl USP 26 Eurotrade, Commerce, S.L; Liquid chromatography–mass spectrometry (LC/MS) grade methanol and water were purchased from Biolab Ltd. Jerusalem.

Formulation preparation

Poly(DL: lactic acid co castor oil) 3:7 designed as p(DLLA-CO) 3:7 with Mn 2200 and Mw 2300 Da was synthesised from castor oil and DL-lactide as previously described.[15] Bupivacaine free base was prepared from bupivacaine hydrochloride by alkaline precipitation and filtration as previously described¹⁵. Bupivacaine free base (15%, w/w) was reduced to 0.25 mm particle size by sieving the powder through a 60-mesh sieve and the powder was incorporated by mixing into the liquid polymer at room temperature to produce a viscous injectable liquid.

Formulation injection

Following the one-week healing period, mice were divided randomly into five groups (n= 28) and bupivacaine-polymer 15% formulation (0.1 ml) was injected via a needle of 22G diameter directly close to the left sciatic nerve using a nerve stimulator (StimuplexR B. Braun Melsungen AG, Germany) at 0.2 mA and 1 Hz to identify the sciatic nerve, under ketamine: xylazine anaesthesia¹⁶.

Animal behaviour

Animal behaviour was monitored followed for signs of toxicity such as weight loss, agitation, malaise, aggression over the 7 days study period.

Sample collection

Three samples were retrieved: plasma, tissue at the injection site and chamber exudate. Samples were obtained at days, 1,2,3,4 and 7 following the bupivacaine-polymer injection.

The exudate sample was drawn from the live non-anaesthetized animal using a 24 G needle that entered directly into an open end of the subcutaneous chamber. The exudate was withdrawn into a tuberculin syringe, typically 0.1 µl-03 µl.

The tissue and plasma samples were taken, following euthanasia (pentobarbital 100 mg/Kg followed by cervical dislocation). Blood samples were drawn from the eye socket, followed immediately by protein precipitation to obtain plasma. Tissue was retrieved from around the sciatic nerve, cleaned, the skin removed, and the sample homogenized.

Analytical methods

For plasma, exudate and tissue were selected according to preliminary tests and expected drug concentrations. LC/MS was selected for plasma and exudate and HPLC for tissue samples. Sample preparation was adapted according to sample size/volume collected and to measurement method selected.

Instrumentation

For plasma and exudates, chromatographic separation of bupivacaine and the internal standard, lidocaine (IS), were performed using a Shimadzu (Kyoto, Japan) UHPLC System, series Nexera, consisting of a CBM-20A LITE controller, two LC-30AD pumps, a Prominence DGU-20A5R degasser, a SIL-30AC autosampler and a CTO-20AC column oven. A C18, 2.6 µm (100 Å pore size, 50 × 2.1 mm) column, Kinetex (Phenomenex, USA) was protected by a SecurityGuard™ (Phenomenex, USA) ULTRA cartridges (C18, 2 × 2.1 mm). MS System: AB SCIEX Triple Quad™ 5500, positive mode. Bupivacaine tissue concentrations were determined using HPLC with a C18 reverse phase column Luna (5 µm, 250 × 4.6 mm).

Data acquisition and analysis of the LC/MS system were performed on a Dell OptiPlex 960 computer with Analyst 1.6 software, AB Sciex. Quantitative calibration (0-30 ng/ml bupivacaine) was performed before every batch of samples. The chromatographic separation was achieved using a gradient method with a mobile phase (MP): [A= 0.1% FA in water, B=0.1% FA in Acetonitrile (ACN)] at a flow rate of 0.45 ml/min over a total run time of 8.5 min.

Linearity range is 0.3-30 ng/ml. Chromatographic oven temperature was set at 40°C. Injection volume: 1 µl. Retention time 2.3 minutes, lidocaine; 2.56 minutes bupivacaine (Figure 2). The chromatographic data were automatically processed for peak-height ratios of the analytes to the IS and fitted to a weighted linear regression. No significant interfering peaks were found at the retention time of bupivacaine and the IS. A limit of quantification (LOQ) 0.3 ng/ml $r^2=0.9989$ with good linearity correlation, was obtained for a high concentration range, accuracy $99.66 \pm 6.48\%$.

Preparation of plasma samples

For each evaluation point, 50 µl of plasma were mixed with 150 µl of water, 50 µl of IS

solution, and 50 µl of MP. Protein precipitation was obtained by addition of 600 µl of ACN, followed by centrifugation at 12000 rpm for 10 min at 4°C. The resultant clear supernatant was filtered through a 0.22 µm filter and analysed by LC/MS. For day 1 and 2, plasma samples were dissolved 1:4 with water. The diluted solution was processed as described above.

Preparation of exudate samples

Exudates samples were evaluated in a similar manner as for plasma. For each evaluation time-point 25 µl of exudates were mixed with 75 µl of water, 25 µl of IS solution and 25 µl of MP. Protein precipitation was obtained by addition of 300 µl of ACN and centrifuge at 12000 rpm for 10 min. Supernatant clear solution was filtered and analysed by LC/MS.

Standard solutions and calibration curves for plasma and exudates samples

Stock solutions of bupivacaine were prepared in formic acid (0.1%) 50%: ACN 50% and stored at 4°C. Lidocaine stock solution was prepared in water to a final concentration of 2.5 µg/ml and used as IS with a final concentration of 138 ng/ml. Each concentration was prepared in duplicate. 100 µl of plasma were diluted 1:2

with double distilled water (DDW) and 50 μ l of the bupivacaine solution and 50 μ l of lidocaine 2.5 μ l/ml were added. Drugs were extracted with 600 μ l of ACN, centrifuged 10 minutes at 12000 rpm, and filtered through a 0.22 μ m filter. For recovery calculation, 200 μ l of water was used.

Bupivacaine was detected using positive ion mode using electrospray ionisation (ESI) and multiple reaction monitoring (MRM) mode of acquisition. Optimal detection conditions were determined by constant infusion of a 100 ng/ml solution of Bupivacaine in 9:1 water: MeOH using the integrated syringe pump (5 μ L/min). The molecular ion of [Bupivacaine+H]⁺ (m/z 289.2) was selected in the first mass analyser and fragmented in the collision cell, followed by detection of the products of fragmentation in the second mass analyser. One transition was monitored for bupivacaine: m/z 289.2 \rightarrow 140.2. Table 1 presents the collision energies and collision cell exit potentials (CXP) for bupivacaine and lidocaine transitions.

Preparation of tissue samples

All tissue samples were suspended in buffer phosphate 0.5% (120 mg/ml) vortexed for 10 min and homogenised. To 500 μ l of the

homogenate, 100 μ l of IS solution were added. Protein precipitation was obtained by addition of 4.2 ml of Isopropanol (IPA), followed by centrifugation at 12000 rpm for 40 min at 4°C. 2.4 ml of the resultant clear supernatant was evaporated to dryness and reconstituted in 200 μ l of buffer phosphate 0.01M pH 3 and filtered through a 0.22 μ m filter. Samples were analysed by HPLC using a Luna 5 μ m C18 column. A mixture of 75% buffer phosphate 0.01M pH 3: 25% ACN at a flow rate of 1 ml/min was used as eluent with UV detection at 210 nm (injection volume 50 μ l, running time 16 minutes). Retention times were 5.15 minutes lidocaine and 13.2 minutes bupivacaine (Figure 3).

A linear calibration plot for assay method was obtained over the calibration range, with a correlation coefficient of 0.9891. The results showed an excellent correlation between the peak area and concentration of the analyte. LOQ was determined as signal-to-noise ratio of 10:1¹⁷. Calibration curves for bupivacaine in release medium were obtained by programmed injection of different aliquots (50 μ l) of a standard solution in concentrations from 0-160 μ g/ml.

Standard solutions and calibration curves for tissue samples

Stock solutions of bupivacaine were prepared in buffer phosphate 0.5% and stored at 4°C. Lidocaine stock was prepared in water (250 ug/ml) and used as IS. Each concentration was prepared in duplicate. 300 mg of tissue was suspended in 1 ml of bupivacaine solution (at different concentrations) and the volume completed to 2.5 ml with buffer phosphate pH 3, and vortexed for 10 minutes. Later, the tissue was homogenised with a tissue homogeniser at 12000 rpm, and then 100 µl of lidocaine were added to 500 µl of the homogenate. Drugs were extracted with 4.2 ml of IPA, homogenised again, centrifuged 40 minutes at 12000 rpm at 4°C and filtered through a 0.22 µm filter. Of the supernatant, 2.4 ml were evaporated to dryness and re-suspended in 200 µl of buffer phosphate pH 3. DDW (300 µl) was used to calculate recovery. MP: 75% Buffer Phosphate 0.01M pH 3: 25% ACN (injection volume 50 µL, run time 16 minutes). Drug concentrations were calculated according to the ratio of IS: bupivacaine and recovery was calculated according to tissue:water concentrations. The limit of detection (LOD) was calculated as signal-to-noise ratio of 3:1, and the LOQ

was determined as signal-to-noise ratio of 10:1 Recovery $85 \pm 0.09\%$.

RESULTS

Following implantation of the titanium subcutaneous chamber, all mice recovered well throughout the 7 days experimental period. Animal weight increased normally during the healing period after chamber placement, and animals did not demonstrate signs of toxicity.

The maximal concentrations of drug observed in plasma, tissue and exudate samples were observed 24 h following site-directed injection of the bupivacaine-polymer formulation. The drug concentrations followed a similar distribution pattern in both plasma and exudate samples with highest concentrations observed during the first 24 h. Bupivacaine concentrations fell during the experimental period (Figure 4). Concentrations of bupivacaine in the exudate samples at each evaluation point were about half of the drug concentration measured in plasma.

The bupivacaine concentrations measured in the extracted tissue samples revealed maximal concentrations 24 h following the injection, as shown for plasma and exudate.

At this evaluation point, the maximum concentration of bupivacaine measured in tissue from the injection site was 7.03 ± 2.75 ug/g. After 48h, bupivacaine concentrations in tissue from the injection site decreased to 54.04 ± 11.28 ng/g. By 72 hours following injection the remaining concentrations at the injection site were 15.71 ± 8.2 ng/g. After this time, the concentrations were below the limit of detection. Drug concentrations were almost undetectable by 72 h following injection.

Comparison of the non-injected right leg did not reveal measured drug concentration at any evaluation point in the tissue samples. The concentrations of drug found in tissue were higher than those found in plasma and exudates.

DISCUSSION

In this investigation, we measured a bupivacaine-polymer formulation assay *in vivo* from an exudate sample collected from the injection site in addition to standard plasma and tissue assay. We observed maximal concentrations of drug samples 24h following site-directed injection. Toxicity of bupivacaine has been associated with drug concentrations in plasma and the need for

animal models to study this effect is well reported^{18,19}.

Dal Bo et al.²⁰ described a similar method to analyse lidocaine concentrations in plasma, using bupivacaine as IS. Lidocaine concentrations of the interstitial fluid near the sciatic nerve were evaluated relative to tissue drug concentration after implantation of extended release formulation²¹. Our results suggest that there was no interference from the plasma matrix, and support determination of biological fluid assays using these methods. The relationship between exudate drug concentrations and plasma concentrations confirms that exudate sampling may be adapted in future studies for pharmacokinetic analysis.

In our investigation, despite the longer half-life of the bupivacaine-polymer (14 h) relative to commercial plain bupivacaine (3 h), the clearance from the injection site was faster than expected. This may be related to the volume of tissue analysed. The site-directed injection was guided using nerve stimulation to locate the nerve; however, this apparatus does not allow precise identification of this tissue for sample collection. The injection site was marked in order to specify the zone for tissue sample

collection, however if smaller focused tissues samples could be collected, potentially higher drug concentrations could be identified at the site of injection. Alternatively, redistribution of the drug in the area surrounding application may explain this finding.

The use of a comparison leg without measured drug concentration at any evaluation point in the tissue samples could be used to understand drug redistribution at the injection site.

Our finding that tissue drug concentrations were higher than those found in plasma and exudates, may be addressed through further dilution of the samples in order to achieve suitable concentrations for LC/MS and to avoid saturation of the column. Using one single measurement method can circumvent the need for two different columns, one for HPLC and one for LC/MS, and reduce differences when comparing results.

One limitation of selecting LC/MS as the best analytical method in our study was the requirement for different dilutions for each sample type to quantify the desired concentrations while avoiding saturation of the measurement system. The measurement

technique utilised may produce different results: HPLC has a different sensitivity than mass spectrometry. LC/MS is a very sensitive measuring technique and this property facilitates detection of very low drug concentrations, thus if low drug concentrations are expected in the site to be tested, LC/MS is a suitable method. Conversely, use of LC/MS to detect high concentrations reduces sensitivity of the measure as the column system can be easily saturated. HPLC is a simple less costly technique that is more suited for higher concentrations. As we expected to find high concentrations of drug in the tissue (>100 ug/g) we selected HPLC to measure tissue samples. Our results suggest that LC/MS was a suitable technique for all our samples given the lower concentrations seen in the tissues. Use of the same method to measure all samples will enable higher sensitivity and avoid the need to develop different methods that each require calibration. Use of radioactive-labelling of the drug in the formulation may enable further understanding of the drug journey in exudate, tissues and plasma following injection of the formulation^{22,23}. We limited the sample size as animals were euthanised at each evaluation point in order to obtain plasma and tissue samples that could be

compared to the exudates. Finally, we measured the total concentration of bupivacaine in plasma rather than free concentration.

CONCLUSION

The drug assays we performed in the current study suggest a novel method to measure drug distribution in exudate samples in the live animal, corresponding to measures of plasma and tissue samples following site-directed injection. This may enable a simple measurement tool that could be used for pharmacokinetic analysis in clinical studies, without requiring sacrifice to retrieve the tissue samples. We were able to detect low drug concentrations due to the very low sensitivity (LOD 0.3 ng/ml). We demonstrated that drug distribution was comparable following the release from the injected site. The subcutaneous chamber with exudate sampling could be used as a measurement tool for pharmacokinetic drug analysis in the live animal.

DECLARATION OF INTERESTS

The authors have no competing interests to declare.

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MRM transitions	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	CXP (V) collision cell exit potential	CE (eV) collision energy
Lidocaine	234.9	86.3	6	45
Bupivacaine	289.2	140.2	12	29

Q1: Quadrupole 1; *m/z*: Mass to charge ratio; Q3: Quadrupole 3; CXP: Collision cell exit potential; CE: Collision energy; V: Volts; eV: Electron volts

Table 1: Multiple reaction monitoring (MRM) transitions and parameters for bupivacaine and lidocaine (IS).

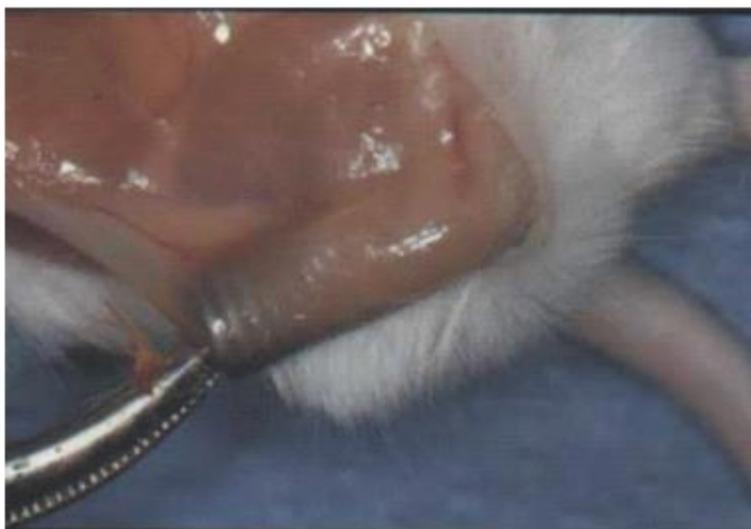


Figure 1: Titanium chamber implanted in the subcutaneous tissue in the dorso-lumbar area of the mouse model.

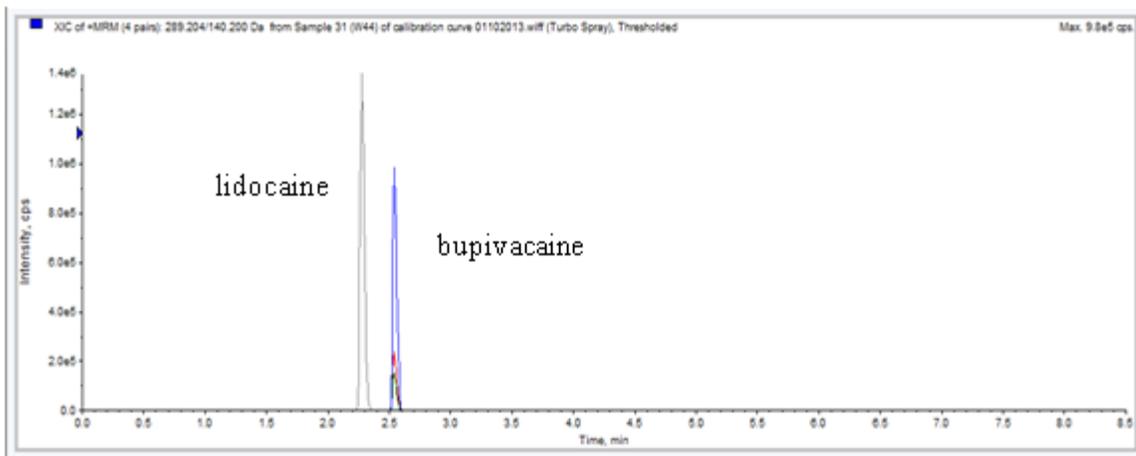


Figure 2: Retention time of bupivacaine and the internal standard lidocaine, as analysed by liquid chromatography–mass spectrometry. A limit of quantification 0.3 ng/ml, $r^2=0.9989$ with good linearity correlation was obtained for a high concentration range. Accuracy $99.66 \pm 6.48\%$.

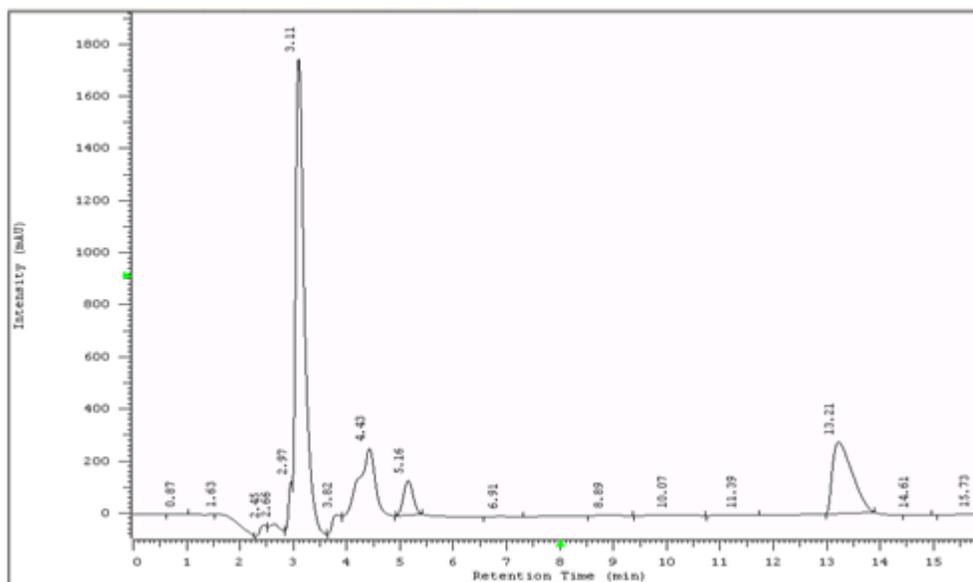


Figure 3: Representative HPLC chromatogram of a tissue sample extracted after injection of p(DLLA-CO) 3:7 loaded with 15% bupivacaine. Retention time of bupivacaine 13.21 min, and the internal standard lidocaine 5.16 min.

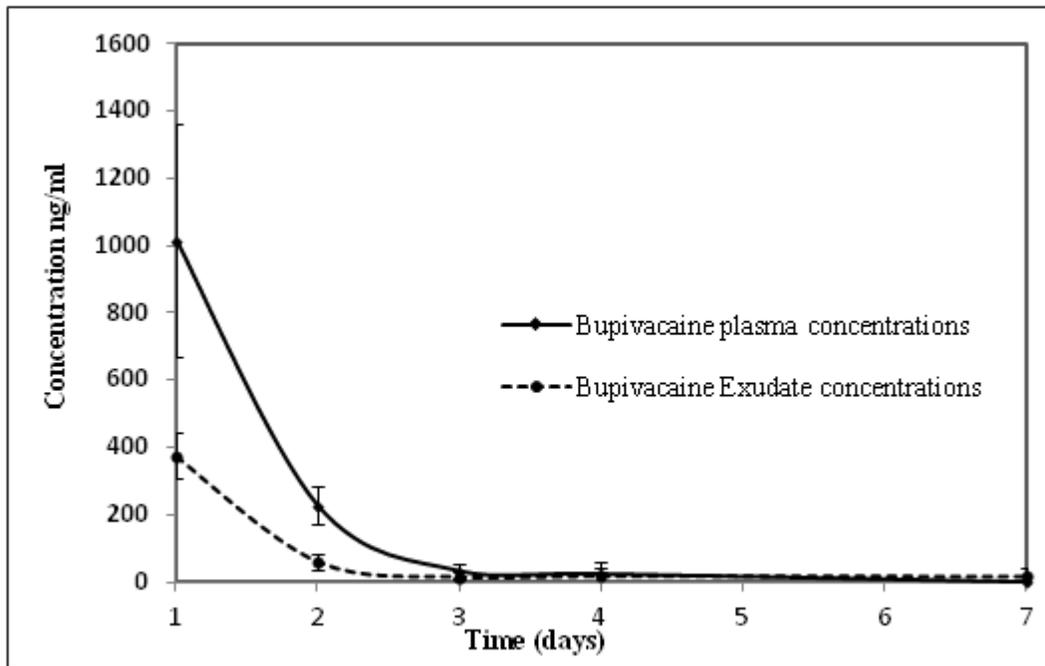


Figure 4: Elimination of bupivacaine from plasma and exudate following a single injection of 0.1 ml of 15% bupivacaine-polymer formulation (for each evaluation point n=5, total n=28).