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Encapsulation of Local Anesthetic Bupivacaine in Biodegradable Poly(DL-lactide-*co*-glycolide) Nanospheres: Factorial Design, Characterization and Cytotoxicity Studies

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Summary: Local anesthetic agents cause temporary blockade of nerve impulses productiong insensitivity to painful stimuli in the area supplied by that nerve. Bupivacaine (BVC) is an amide-type local anesthetic widely used in surgery and obstetrics for sustained peripheral and central nerve blockade. In this study, we prepared and characterized nanosphere formulations containing BVC. To achieve these goals, BVC loaded poly(DL-lactide-*co*-glycolide) (PLGA) nanospheres (NS) were prepared by nanopreciptation and characterized with regard to size distribution, drug loading and cytotoxicity assays. The 2^{3-1} factorial experimental design was used to study the influence of three different independent variables on nanoparticle drug loading. BVC was assayed by HPLC, the particle size and zeta potential were determined by dynamic light scattering. BVC was determined using a combined ultrafiltration-centrifugation technique. The results of optimized formulations showed a narrow size distribution with a polydispersivity of 0.05%, an average diameter of 236.7 ± 2.6 nm and the zeta potential $-2.93 \pm 1{,}10$ mV. In toxicity studies with fibroblast 3T3 cells, BVC loaded-PLGA-NS increased cell viability, in comparison with the effect produced by free BVC. In this way, BVC-loaded PLGA-NS decreased BVC toxicity. The development of BVC formulations in carriers such as nanospheres could offer the possibility of controlling drug delivery in biological systems, prolonging the anesthetic effect and reducing toxicity.

Keywords: bupivacaine; local anesthetic; polymeric nanoparticle; poly(DL-lactide-*co*-glycolide) nanospheres

Introduction

Local anesthetics (LAs) are small molecules that are rapidly redistributed from the site of injection, limiting the duration of analgesia. In the clinical setting, local anesthetics are employed in various techniques to produce local or regional anesthesia and analgesia. LAs have considerable side effects on the central nervous system and the cardiovascular system when accidentally injected intravascularly or administered in excessive doses.[1–3]

Bupivacaine, a long acting local anesthetic of amide-type, is widely used mainly for regional nerve blockade, particularly when a prolonged effect is required. Its structure contains a single chirality centre; it is used as the racemate of (-) - (*R*) and (-) - (*S*)-bupivacaine (*rac*-bupivacaine). Both

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enantiomers are active; however, a longer duration of neural blockade as well as lower propensity to toxicity are produced by (-) - (*S*)-bupivacaine.[4–6]

This fact has led to the introduction of bupivacaine with enantiomeric excess of (*S*)-isomer into clinical practice under the name of new bupivacaine.[7] Its safety and efficacy have been compared with that of *rac*-bupivacaine in surgical anesthesia and pain management.[7] Nowadays, there is a strong clinical requirement for long-acting anesthetics as well as for LAs with low systemic uptake, leading to less toxic side effects.[8,9]

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. The development of local anesthetic formulations in carriers such as liposomes,[10] cyclodextrins,[11] biopolymers,[12] microspheres,[13] nanoparticle[14–16] among others, could offer the possibility of controlling drug delivery in biological systems, prolonging the anesthetic effect and reducing its toxicity. Polymeric nanospheres composed by biocompatible and biodegradable compound have lower toxicity compared with other polymers and are more stable than liposomes, solid lipid nanoparticles, microspheres and other drug delivery systems in biological environments. PLGA is composed of lactic and glycolic acid units linked together by ester bonds. The polymer degradation proceeds with the formation of free carboxylic end groups. Several current reports have indicated the presence of low microenvironmental pH in large samples of PLGA.[17–20]

The commonly reported methods of preparing nanoparticles of biodegradable polymers include solvent evaporation, monomer polymerization, nanoprecipitation and salting-out procedures.[21] The nanoprecipitation method developed by Fessi et al.[22] is an easy and reproducible technique, very often used for preparation of colloidal carriers such as nanospheres and nanocapsules. The method was based on the interfacial deposition of a polymer followed by diffusion of a semipolar and miscible solvent in aqueous medium in the presence of a surfactant.[23]

Several important factors contribute to the effectiveness of this method of preparation of particles with an acceptable size range, shape and the percentage of the drug loading, namely the amount of polymer, surfactant percentage, volume of organic phase and others.[24–26] It is difficult to assess the effect of the variables individually or in combination, therefore derivation of a mathematical model suitable for establishing a quantitative relationship between the formulation variables is necessary.[24–26]

The aim of the present study was to prepare new BVC (25% (*R*) - 75% (*S*)) loaded nanoparticles by the nanoprecipitation method. The 2^{3-1} factorial experimental design was used to study the influence of three different independent variables on nanoparticle drug loading. The advantage of BVC incorporation in nanospheres instead of microspheres was in that the traditional drug microencapsulation methods do not provide monodisperse particles. The resulting BVC nanoparticles were characterized with regard to size distribution, drug loading, in vitro drug release and cytotoxicity assays.

Experimental Part

Reagents and Chemicals

Poly(DL-lactide-*co*-glycolide) (MW 60000) and poly(vinyl alcohol) (PVA) were supplied by Sigma Chem Co. BVC was kindly given by Cristália (Itapira, Brazil). All other chemicals were reagent grade.

Preparation of Nanospheres

BVC-loaded PLGA nanospheres were prepared using nanoprecipitation procedure described earlier.[22] PLGA and BVC (0.25%) was dissolved in acetone and immediately poured into stirred aqueous solution (20 mL) of phosphate buffer (pH 7.4, 5 mM) containing PVA as a hydrophilic surfactant. The resulting cloudy suspension was quickly transferred to a

round-bottomed flask and the solvent evaporated under reduced pressure for 20 min at 58 °C and the final volume of the aqueous suspension was adjusted to 10 mL. The quantities of the substances were defined by experimental design.

Experimental Design

Based on preliminary study of the effect of parameters on the drug loading of nanospheres, the experiments were performed by nanoprecipitation using a two-level factorial design. Design-expert version 6 software was applied to designing the experiment. Three variables were taken at two levels, low and high, which were represented by transformation values of −1 and +1, respectively. Values of these selected variables are shown in Table 1.

The analysis of factorial design experiments contrast was realized with Minitab 15.1 software with $p < 0.05$ of significance.

Physicochemical Characterization of Suspensions

Particle Size and Zeta Potential

Dynamic light scattering (Brookhaven Instruments Corp., Holtsville, NY) was used to measure the average nanoparticles size and size distribution (polydispersity index). All measurements were done at 25 °C with an angle detection of 90°. The zeta potential of nanoparticles was measured on a Zeta plus analyzer (Brookhaven Instruments Corp., Holtsville, NY). All preparations were diluted at 1:20 with deionized water and measured in triplicate.

Drug Immobilization Efficiency

To determine entrapment efficiency in nanoparticles, the BVC-loaded PLGA-NS was centrifugated for 20 min at 200 *g* using ultrafiltration/centrifugation (Ultrafree-MC 30 000 MW, Millipore). Free non-associated BVC was determined in the ultrafiltrate after separation of the nanoparticles. 100% of BVC concentration was determined after dissolution of dispersions with acetonitrile. The amount of BVC was measured by a validated HPLC method.[27] The entrapment efficiency (EE, %) of BVC was determined from the difference between the total (measured in dispersions) and the free drug (measured in the ultrafiltrate) concentrations according to Eq. 1.

$$\mathrm{EE}(\%) = \frac{W_s}{W_{total}} x100\% \quad (1)$$

where W_s is the amount of BVC in PLGA-NS and W_{total} is the amount of BVC used in formulation.

Cell Culture and Cytotoxic Assays

3T3 cells were seeded onto 96-well microtiter plates (0.1 ml/well, containing 2.10^4 cells/well) and cultured (for 48 h) in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, 100 UI/mL penicillin and 100 μg/mL streptomycin sulfate (pH 7.2–7.4) in humid atmosphere, at 37 °C and 5% CO_2. Test chemicals (BVC, PLGA-NS and BVC-PLGA-NS) were incubated at two concentrations (0.25% and 1.25%). The cells were exposed to increasing concentrations of the chemicals for 24 h. After incubation, cell viability was assessed by tetrazolium reduction (MTT test). Cellular damage results in loss of the metabolic cell function. The tetrazolium salt MTT is widely used to quantify by colorimetric assay the cytotoxicity of preparations. The tetrazolium salts are metabolically reduced to highly colored

Table 1.
Values and coded units of 2^{3-1} factorial design for preparation of BVC nanoparticles by nanopreciptation.

Variables	Coded units	Levels		Formulation	A	B	C
		−	+				
				1	+	−	−
PLGA (mg)	A	25	60	2	−	−	+
Organic phase (mL)	B	10	25	3	−	+	−
PVA (mg)	C	30	100	4	+	+	+

end products called formazans. The colorless MTT is cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain and is active only in viable cells. Briefly, 1 mg/mL MTT solution was added into each well and the plate was incubated for another 1 h. Following incubation, culture medium was removed from wells and formazan crystals formed were dissolved in HCl - isopropyl alcohol mixture (1:24 v/v) and shaken at room temperature for 20 min. The absorbance of each well was measured using a microculture plate reader at 492 nm. Cell viability was expressed as a percentage of the control group.[14] Cytotoxic data were analyzed by one-way analysis of variance (one-way ANOVA), with Tukey-Kramer as a post hoc test. Statistical significance was defined as $p < 0.001$.

Results and Discussion

Preparation and Characterization of Nanoparticles

A technique of two-level factorial design offers the possibility of investigating four independent variables at two levels after performing only sixteen experiments. The selection of factors and levels in the design, which most affects drug loading, would be based on the results of preliminary investigations.

The nanoparticle preparation by nanoprecipitation, apparently simple, may involve complex interfacial hydrodynamic phenomena. Addition of acetone-polymer solution resulted in spontaneous emulsification of the polymer solution in the form of nanodroplets due to some kind of interface instability arising from rapid diffusion of acetone across the interface and a marked decrease in interfacial tension. The mechanism of nanosphere formation could be explained in terms of interfacial turbulence or spontaneous agitation of the interface between two unequilibrated liquid phases, involving flow, diffusion and surface processes.[22]

Primary independent factors that might affect drug loading in nanoprecipitation are: amount of polymer (A), amount of surfactant (B) and volume of organic phase (C). We evaluated the influence of these parameters on drug loading by a factorial experimental design. Four batches of different combinations were prepared by taking values of selected variables - A, B, C - at two levels as shown in Table 1. The variation of the size of nanoparticles, zeta potential, polydispersivity and BVC-loaded PLGA-NS obtained for the factorial design is shown in Table 2.

Based on the results of highly drug-loaded nanoparticles the optimum setting was significantly ($p < 0.05$) affected by the volume of organic solvent (acetone - factor B) and the amount of PVA (factor C). As shown in Table 2, the optimum formulation with highest drug loading was achieved with 60 mg of polymer, 10 mL of organic phase and 30 mg of emulsifier.

The resulting BVC nanoparticles showed the best response to size at 236 nm, efficiency entrapment ca. 5% and zeta potential around −13 mV, indicating an adequate homogeneity of this system and in agreement with PLGA nanoparticles related in literature.[28,29] The low immobilization efficiency (∼5%) of BVC PLGA-NS was due physical-chemistry characteristics of the BVC molecule. In this

Table 2.
Experimental design and physicochemical characteristics of PLGA-NS loaded with BVC storage at 25 °C (mean ± S.D, n = 2 experiments).

Formulation	A	B	C	Particle size ± S.D (nm)	Polydispersivity ± S.D.	Zeta potential ± S.D. (mV)	Drug loading ± S.D. (%)
1	+	−	−	236 ± 02	0.069 ± 0.016	−12.93 ± 1.10	5.11 ± 0.091
2	−	−	+	214 ± 01	0.079 ± 0.014	−6.53 ± 0.84	4.89 ± 0.738
3	−	+	−	152 ± 02	0.090 ± 0.012	−12.19 ± 2.70	4.01 ± 0.581
4	+	+	+	204 ± 02	0.099 ± 0.020	−9.05 ± 1.52	2.28 ± 0.193

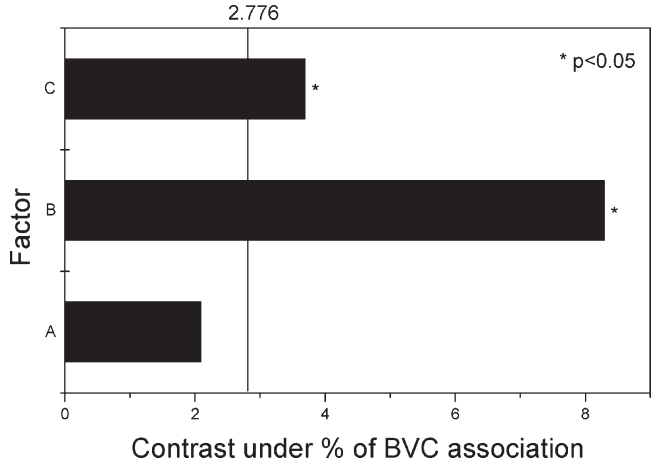

Figure 1.
Contrast values of factors A, B and C on the % of BVC loading in PLGA nanospheres.

study BVC was used as hydrochloride form, i.e., with good water solubility (30 mM). The literature described that PLGA-NS presents low drug incorporation efficiencies, especially of water-soluble drugs, due to their small size and hence large surface area, which promotes drug loss into the aqueous phase during the particle formation.[30] Similar results of BVC were found for local anesthetics procaine and ropivacaine in nanoparticles formed by copolymers, where the EE (%) was ca. 6%[30] and 3%, respectively.[7,14]

Cell Culture and Cytotoxic Assays

The cytotoxicity of BVC and BVC-PLGA-NS (formulation 2, Table 2) in the same proportions (0.25 and 1.25%) was measured using 3T3 cell cultures. Cell proliferation was determined using MTT assay. Figure 2 shows the effect of concentration on the 3T3 cell viability, with significant

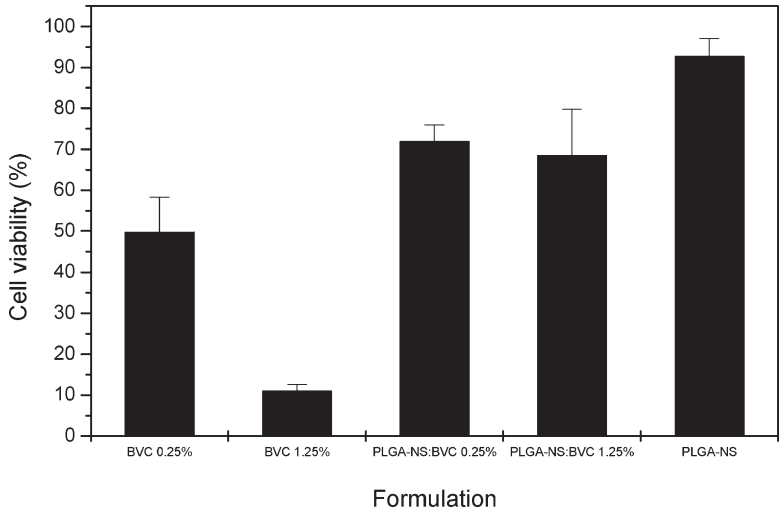

Figure 2.
Cytotoxic effects of BVC, PLGA-NS, PLGA-NS:BVC at 0.25% and 1.25% on Balb/c 3T3 cells incubated for 24 h at 37 °C and 5% CO_2 as evaluated by the MTT reduction test. Data expressed as % cell viability (Mean ± SD, $n = 8$ experiments). $p < 0.001$ (one-way ANOVA with Tukey-Kramer *post hoc* test).

differences between the results for BVC and the BVC-loaded PLGA nanospheres.

When fibroblasts were treated with BVC at the concentration of 1.25%, the percentage of viable cells was reduced near 10% and is presented as dose-response data (Figure 2). Figure 2 showed that PLGA-NS had a very little effect on the cell viability and that BVC loaded PLGA-NS induced a maximum concentration tested (1.25%) comparable to that of PLGA-NS, i.e., this formulation was less toxic than free BVC ($p < 0.001$). The results of BVC-loaded PLGA-NS formulation showed no dose-response data when compared with free BVC (in the range of concentration tested), indicating that the cellular protective effects observed on treatment with the BVC-loaded PLGA-NS could be explained by the sustained release of BVC from the nanospheres. The evaluation of concentration up to used in clinical procedures (0.25 and 0.5%) was to show that BVC-loaded PLGA-NS formulation increased the clinical security of this local anesthetic.

Conclusion

In the present study, the preparation and characterization of nanoparticles of BVC showed that BVC loaded PLGA-NS are stable, decreasing the cytotoxicity of BVC compared with neat BVC. The influence of different factors on the properties of the nanoparticles was optimized using a 2^{3-1} factorial experimental design. The polymer amount and both external and internal phase volumes had a statically significant difference on the drug loading. The optimum formulation with the highest drug loading was achieved with 60 mg of the polymer, 10 mL of organic phase and 30 mg of emulsifier. In general the results show that the PLGA nanoparticles can be considered as a promising carrier system for controlled release of BVC with possible clinical applications for pain treatment.

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[1] D. R. de Araújo, E. de Paula, L. F. Fraceto, *Quim. Nova* **2008**, *31*, 1775.
[2] R. H. de Jong, *Local anesthetics*, C.C. Thomas, Springfield 1994.
[3] J. M. Ritchie, G. R. Strichartz, *Local Anesthetics - Handbook of Experimental Pharmacology*, Springer-Verlag, Berlin 1987.
[4] L. E. Mather, P. McCall, P. L. McNicol, *Anesth. Analg.* **1995**, *80*, 328.
[5] R. W. Gristwood, *Drug Saf.* **2002**, *25*, 153.
[6] Y. F. Huang, M. E. Pryor, L. E. Mather, B. T. Veering, *Anesth. Analg.* **1998**, *86*, 797.
[7] R. H. Foster, A. Markham, *Drugs* **2000**, *59*, 551.
[8] L. E. Mather, P. McCall, P. L. McNicol, *Anesth. Analg.* **1995**, *80*, 328.
[9] D. R. de Araújo, L. F. Fraceto, A. F. A. Braga, E. de Paula, *Rev. Bras. Anestesiol.* **2005**, *55*, 316.
[10] L. M. A. Pinto, D. K. Yokaichiya, L. F. Fraceto, E. de Paula, *Biophys. Chem.* **2000**, *87*, 213.
[11] L. M. A. Pinto, L. F. Fraceto, M. H. A. Santana, T. A. Pertinhez, S. Oyama-Junior, E. de Paula, *J. Pharm. Biomed. Anal.* **2005**, *39*, 956.
[12] M. Polakovic, T. Gorner, R. Gref, E. Dellacherie, *J. Controlled Release* **1999**, *60*, 169.
[13] M. D. Blanco, M. V. Bernardo, R. L. Sastre, R. Olmo, E. Muniz, J. M. Teijón, *Eur. J. Pharm. Biopharm.* **2003**, *55*, 229.
[14] C. M. Moraes, A. P. de Matos, R. Lima, A. H. Rosa, E. de Paula, L. F. Fraceto, *J. Biol. Phys.* **2007**, *33*, 455.
[15] T. Govender, S. Stolnik, M. C. Garnett, L. I. Stanley, S. Davis, *J. Controlled Release* **1999**, *57*, 171.
[16] T. Gorner, R. Gref, D. Michenor, F. Sommer, M. N. Tran, E. Dellacherie, *J. Controlled Release* **1999**, *57*, 259.
[17] S. P. Schwendeman, M. Cardamone, A. Klibanov, R. Langer, S. Chen, H. Bernstein, *Microparticulate Systems for the Delivery of Proteins and Vaccines*, Marcel Dekker, New York 1996.
[18] A. Shenderova, T. G. Burke, S. P. Schwendeman, *Pharm. Res.* **1997**, *14*, 1406.
[19] A. Shenderova, T. G. Burke, S. P. Schwendeman, *Pharm. Res.* **1999**, *16*, 241.
[20] S. R. Mallery, A. Shenderova, P. Pei, S. Begum, J. R. Ciminieri, *Anticancer Res.* **2001**, *21*, 1713.
[21] D. Quintanar-Guerrero, E. Allemann, H. Fessi, E. Doelker, *Drug Dev. Ind. Pharm.* **1998**, *24*, 1113.
[22] H. Fessi, F. Puisieux, J. P. Devissaguet, N. Ammoury, S. Benita, *Int. J. Pharm.* **1989**, R1.
[23] J. M. Barichello, M. Morishita, K. Takayama, T. Nagai, *J. Drug Dev. Ind. Pharm.* **1999**, *25*, 471.

[24] S. C. Yang, J. B. Zhu, *Drug. Dev. Ind. Pharm.* **2002**, *28*, 265.
[25] G. A. Lewis, *Encyclopedia Pharm. Technol.* **2002**, *2*, 1922.
[26] A. K. Seth, A. Misra, *J. Pharm. Pharm. Sci.* **2002**, *5*, 285.
[27] C. M. Moraes, A. H. Rosa, E. de Paula, L. F. Fraceto, *Quim. Nova* **2008**, *31*, (in press).
[28] K. Avgoustakis, A. Beletsi, Z. Panagi, P. Kleptsanis, E. Livaniou, G. Evangelatos, D. S. Ithakissios, *Int. J. Pharm.* **2003**, *259*, 115.
[29] C. B. Michalowski, S. S. Guterres, T. Dalla Costa, *J. Pharm. Biomed. Anal.* **2004**, *35*, 1093.
[30] T. Govender, T. Riley, T. Ehtezazi, M. C. Garnett, S. Solnik, L. Illum, S. S. Davis, *Int. J. Pharm.* **2000**, *199*, 95.

