Revista de Ciências Farmacêuticas Básica e Aplicada
Journal of Basic and Applied Pharmaceutical Sciences

Rev. Ciênc. Farm. Básica Apl., v. 27, n.3, p.207-212, 2006
ISSN 1808-4532

Inclusion complex of S(-) bupivacaine and 2-hydroxypropyl- β-cyclodextrin: study of morphology and cytotoxicity

Moraes, C.M.[1]; Araújo, D.R.[2]; Issa, M.G.[3]; Ferraz, H.G.[3]; Yokaichiya, F.[4]; Franco, M.K.K.D.[4]; Mazzaro, I.[4]; Lopes, P.S.[1]; Gonçalves, M.M.[1]; de Paula, E.[2], Fraceto, L.F.[1,2,5]*

[1]*Department of Biochemistry, University of Sorocaba, UNISO, Sorocaba, SP, Brazil.*
[2]*Department of Biochemistry, Institute of Biology, State University of Campinas, UNICAMP, Campinas, SP, Brazil.*
[3]*Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo, USP, São Paulo, SP, Brazil.*
[4]*Departament of Physics, Federal University of Paraná, Curitiba, PR, Brazil.*
[5]*Departament of Biochemisty, State University of São Paulo, UNESP, Sorocaba, SP, Brazil.*

Recebido 06/12/06 / Aceito 10/04/07

ABSTRACT

Local anesthetics (LA) belong to a class of pharmacological compounds that attenuate or eliminate pain by binding to the sodium channel of excitable membranes, blocking the influx of sodium ions and the propagation of the nerve impulse. S (-) bupivacaine (S(-) bvc) is a local anesthetic of amino-amide type, widely used in surgery and obstetrics for sustained peripheral and central nerve blockade. This article focuses on the characterization of an inclusion complex of S(-) bvc in 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). Differential scanning calorimetry, scanning electron microscopy and X-Ray diffraction analysis showed structural changes in the complex. In preliminary toxicity studies, the cell viability tests revealed that the inclusion complex decreased the toxic effect ($p<0.001$) produced by S(-) bvc. These results suggest that the S(-) bvc:HP-β-CD inclusion complex represents a promising agent for the treatment of regional pain.

Keywords: S(-) bupivacaine; cyclodextrin; inclusion complex.

INTRODUCTION

Local anesthetics (LA) belong to one of the classes of pharmacological compounds used to attenuate or eliminate pain. These drugs, which inhibit reversibly the excitation-transmission process in axons, have a relatively short-term action and a significant toxicity to the central nervous and cardiovascular systems (Covino & Vassalo, 1976; Strichartz & Ritchie, 1987; Jong, 1994). Nowadays, there is a strong clinical need for long-acting LA, as well as for drug molecules with decreased systemic uptake that could lead to less toxic side effects.

Bupivacaine is an amino-amide type local anesthetic widely used in surgery and obstetrics for sustained peripheral and central nerve blockade. The molecule has a single chiral centre and the drug is marketed as the racemic mixture of R(+) and S(-) bupivacaine (rac-bupivacaine). Both enantiomers are active but the S(-) form has a longer duration of neural blockade, as well as a lower toxicity towards the central nervous and cardiovascular systems (Mather et al., 1995; Huang et al., 1998; Gristwood, 2002).(Figure 1)

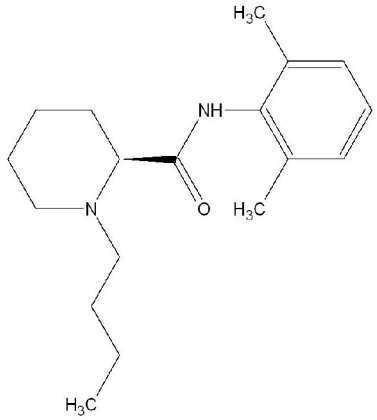

Figure1. Chemical structure of S(-) bupivcaine.

This has led to the introduction of S(-) bupivacaine into clinical practice, under the name levobupivacaine (Foster & Markham, 2000). Its safety and efficacy has been compared with that of rac-bupivacaine in surgical anesthesia and pain management (Foster & Markham, 2000).

Although commercially available local anesthetic formulations are used in a variety of doses and routes of administration, the relatively short duration of action - due to the transfer and redistribution of the agent from the site of injection - restricts their clinical use (Grant & Bansinath, 2001; Rose et al., 2005).

Thus, controlled-release systems would be highly desirable for the clinical use of local anesthetics, offering

* *Corresponding Author*: Leonardo Fernandes Fraceto - Universidade Estadual Paulista - UNESP - Av. Três de Março, 511 - Alto da Boa Vista - CEP: 18087-180 - Sorocaba - SP - Brasil - Telefone: (15) 3238-3415 - Fax: (15) 3228-2842 - e-mail: leonardo@sorocaba.unesp.br

the possibility of prolonging their duration of action and/or reducing their toxicity (Hirayama & Uekama,1999; Araújo et al., 2005; Rose et al., 2005).

Cyclodextrins are cyclic oligosaccharides capable of forming complexes with lipophilic drugs, thus modifying their physicochemical and biopharmaceutical properties (Bibby et al., 2000). The α -, β -, γ -cyclodextrins are widely-used natural cyclodextrins that consist of six, seven and eight D-glucopyranose residues, respectively, linked by α -1,4 glycosidic bonds into a macrocycle with a central cavity. Owing to their ability to alter the physical, chemical, and biological properties of guest molecules occupying the cavity, cyclodextrins are potential candidates for drug delivery devices (Ren et al., 2002).

The principal advantages of natural cyclodextrins as drug carriers are: a well-defined chemical structure with many potential sites for chemical modification or conjugation; the availability of cyclodextrins of varying cavity sizes, low toxicity and low pharmacological activity; some degree of water solubility, and protection of the included/conjugated drugs from biodegradation (Thompson, 1997).

In fact, examples exist in the literature that demonstrate the advantages of controlled release of LA in cyclodextrin, such as prolonged action and reduced toxicity (Araujo et al., 2005; 2006; Pinto et al., 2005). The purpose of the present study was to prepare, characterize and assess the cytotoxic activity of a S(-) bvc inclusion complex with HP-β-CD, in comparison with a plain solution of S(-) bvc. This particular system has clinical relevance enabling the use of S(-) bvc as an alternative means of pain treatment.

MATERIAL AND METHODS

Reagents and chemicals

S(-) Bupivacaine was a gift from Cristália Ind. Farm. LTDA (Itapira, Brazil); HP-β-CD was purchased from Roquette and was characterized as having a degree of substitution of 4.2, based on Fourier-transformed infrared spectrophotometric (FT-IR) analysis (Michaud & Icart, 2001). Sodium dihydrogen phosphate and disodium hydrogen phosphate (anhydrous) were from Sigma Chem. Co. and Deionized water (resistivity 18m .cm) from a Waters ultra pure water system.

Preparation of solid inclusion complex

The inclusion complex was prepared by shaking appropriate weights of the S(-) bvc and HP- β -CD (1:1 molar ratio) in deionized water, at room temperature (25 ± 1 °C) for 24 h. After reaching equilibrium, the solution was freeze-dried in a Freezone® 4.5L freeze-dry system (Labconco) and stored at -20 °C for further use. A physical mixture (uncomplexed drug) was prepared by mixing S(-) bvc and HP-β-CD powders.

Differential Scanning Calorimetry

The various samples (2 mg) were placed in aluminum pans in a DSC2920 TA Instruments calorimeter (University of São Paulo) and heated from 25-300 °C at 10ºC/ min, in nitrogen flowing at 50mL/min. An empty pan served as reference and pure Indium was used to calibrate the temperature. Measurements were performed on the S(-) bvc:HP-β-CD inclusion complex (molar ratio 1:1) and the equivalent physical mixture; S(-) bvc and HP-β-CD thermograms were also run.

Scanning Electron Microscopy

The S(-) bvc:HP-β-CD inclusion complex (1:1 molar ratio) was morphologically analyzed by Scanning Electron Microscopy (SEM), with a JEOL J-210 Scanning Microscope. Samples of S (-) bvc, HP-β-CD and the S(-) bvc:HP-β-CD physical mixture (1:1) were also prepared at the same concentration as the inclusion complex. The samples were mounted on aluminum stubs, using double-sided sticky tabs, and vacuum coated with gold for 180 s, to render them electrically conductive.

X-Ray analysis

Powder diffractograms of HP-β-CD, S(-) bvc, physical mixture and the inclusion complex were collected on a Rigaku wide-Angle goniometer with a Co-Kα radiation source (Philips PW 1743) and voltage and current set to 40 kV and 20 mA, respectively, operated at a scan rate of 1°/ min, between 2θ = 5° and 60° in a θ-2θ configuration with a pyrolytic graphite crystal analyzer.

Cell culture and cytotoxic assays

Balb/c mouse fibroblasts (3T3 cells) were cultured in DMEM (Dulbecco´s Modified Eagle Medium) supplemented with 10% fetal bovine serum, 100UI/mL penicillin and 100 g/mL streptomycin sulfate (pH 7.2-7.4) under a humidified atmosphere, at 37oC and 5% CO_2. Cells were seeded ($2x10^4$ cells/well) in 48-well tissue culture plates and cultured for 48h. The cells were then incubated for 2h with the test compound (S(-) bvc, HP- -CD or complex) at three different concentration 0.2, 0.4 and 0.6 mM. Cell viability was assessed by tetrazolium reduction (MTT test). 1mg/mL MTT was incubated for 1h with the treated 3T3 cells, at 37ºC. The number of viable cells was determined by measuring the amount of MTT converted to insoluble formazan dye by mitochondrial dehydrogenases. The formazan crystals formed were dissolved in a 1 M HCl-isopropyl alcohol mixture (1:24 v/v) and shaken for 20 min at room temperature. Cytotoxic assay 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 $p< 0.05$.

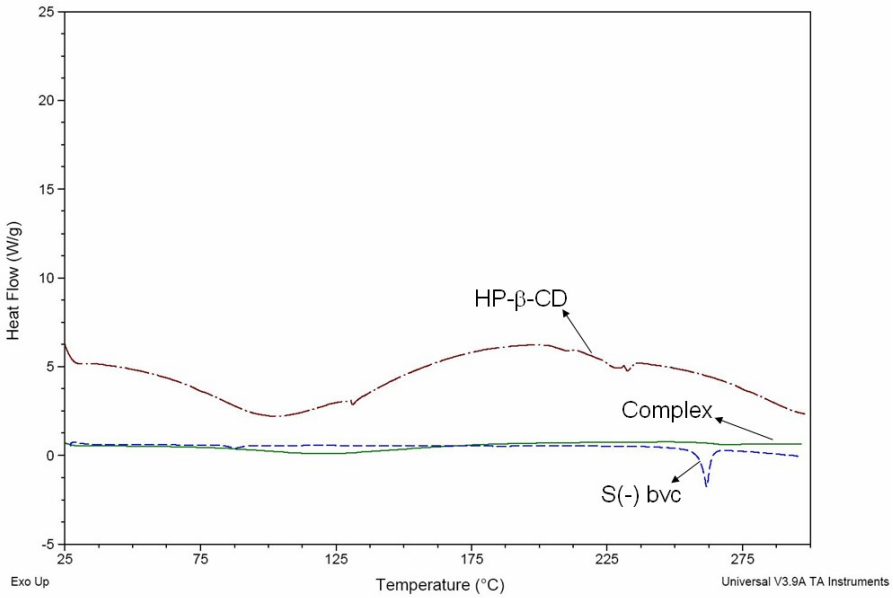

Figure 2. : Differential scanning calorimetry thermograms of HP-β-CD, S(-) bvc and c) S(-) bvc:HP-β-CD 1:1 inclusion complex.

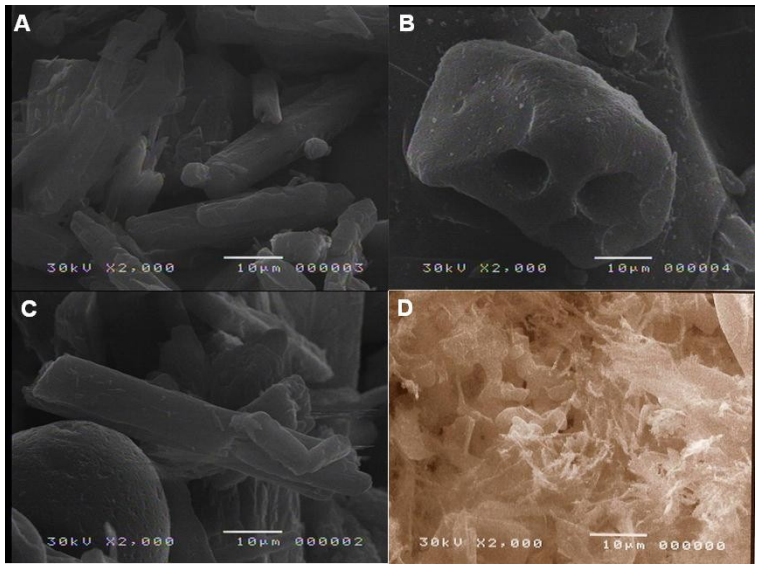

Figure 3. Scanning electron micrographs of (A) S(-) bvc, (B) lyophilized HP-β-CD, (C) physical mixture of S(-) bvc and HP-β-CD(1:1, molar ratio), (D) inclusion complex of S(-) bvc and HP-β-CD (1:1, molar ratio). 2000 x magnification, bar = 10 μm.

RESULTS

Differential Scanning Calorimetry (DSC)

Representative DSC thermograms measuring the rate of heat absorption by S(-) bvc, HP-β-CD, S(-) bvc:HP-β -CD inclusion complex (at 1:1 molar ratio) are shown in Figure 2. The DSC thermogram of free S(-) bvc exhibited an endothermic peak at 262.5 °C, corresponding to the melting point of this compound (Loftsson & Brewster, 1996). The thermogram for HP-β-CD showed one broad endothermic peak near 100°C, corresponding to the release of water from HP-β-CD (Kohata et al., 1993). The thermogram of the S(-) bvc:HP-β-CD physical mixture was similar to that of S(-) bvc, with an endothermic peak at 262.5 (data not shown), whereas this peak was absent from the thermogram for the S(-) bvc:HP-β-CD complex.

Scanning Electron Microscopy (SEM)

Figure 3 shows that the shape and size of the inclusion complex was completely different from those of

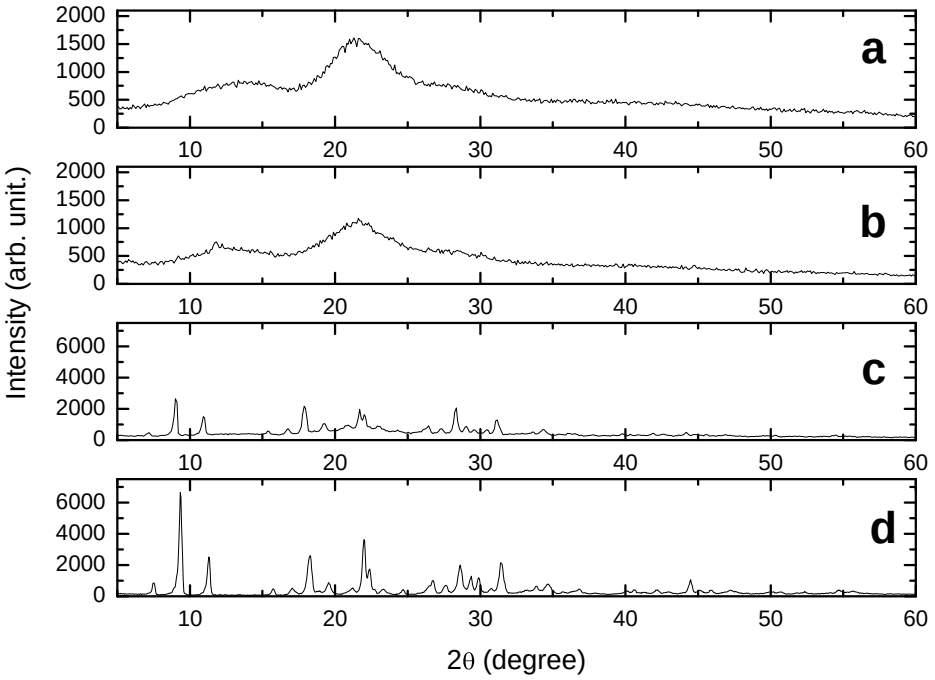

Figure 4. The X-ray powder diffraction patterns of a) HP-β-CD, b) S(-) bvc:HP-β-CD inclusion complex, c) S(-) bvc/HP-β-CD 1:1 physical mixture and d) S(-) bvc.

free S(-) bvc or HP- CD. The physical mixture of S(-) bvc and HP-β-CD powders (Figure 3C) revealed similarities with the crystals of free molecules. At the 1:1 molar ratio of the S(-) bvc:HP-β-CD inclusion complex under study, a compact and homogeneous powder-like structure was observed (Figure 3D) which was smaller than the crystals of S(-) bvc or HP-β-CD alone.

X-Ray analysis

The diffractograms of S(-) bvc, HP- β -CD, physical mixture and inclusion complex are shown in Figure 4. It can be seen that HP- β -CD and the complex gave patterns typical of amorphous material, while samples containing the free drug (Figure 4C and 4 D) showed crystallinity.

Cell culture and cytotoxic assays

Measurement of the effect of S(-) bvc, the complex and HP-β-CD on the 3T3 cell viability is a way to evaluate the cytotoxicity of these chemical substances. The 3T3 cells were treated at three different concentrations (0.2, 0.4 and 0.6 mM) of each compound. Figure 5 shows the effect of concentration on the 3T3 cell viability (%), with significant differences between the results for S(-) bvc and the S(-) bvc:HP-β-CD complex.

DISCUSSION

The use of DSC in combination with other experimental techniques, such as X-ray, infrared, HPLC, UV-Vis, scanning electron microscopy and NMR, is very useful for the study of the properties of inclusion complexes (Naidu et al., 2004). Simultaneous analysis of complexation behavior by these different methods obviously has many advantages, providing definitive experimental evidence for the formation of the drug-cyclodextrin inclusion complex. In the current study, further information about the complex of S(-) bvc with HP-β-CD has been obtained from DSC, SEM, X-Ray data and cytotoxicity assays.

The thermogram for the inclusion complex shows the absence of the characteristic endothermic peak of S(-) bvc at 262.5 ^{o}C (Figure 2), indicating the formation of an amorphous solid dispersion. The disappearance or shifting of endo- or exothermic peaks of drugs is usually an indication of changes in the physicochemical characteristics of the system (Loukas et al., 1997). Here, it shows the absence of uncomplexed S(-) bvc.

Scanning electron microscopy (SEM) can be used to study the microscopic aspects of raw materials (cyclodextrin and drug) and the products obtained by different methods of preparation, such as physical mixture, solution complexation, coevaporation, etc. Although there is a clear difference in the state of crystallization of the raw materials and the products, in these micrographs, this alone is inadequate to affirm inclusion complexation; nevertheless, it helps to assess the existence of a single component in the preparation obtained, such as the inclusion complex in Figure 3.

Powder X-ray diffractometry is a useful method for the detection of cyclodextrin complexation in powder or microcrystalline states. The diffraction pattern of the complex should be clearly distinct from that of the superimposition of each of the components if a true inclusion complex has

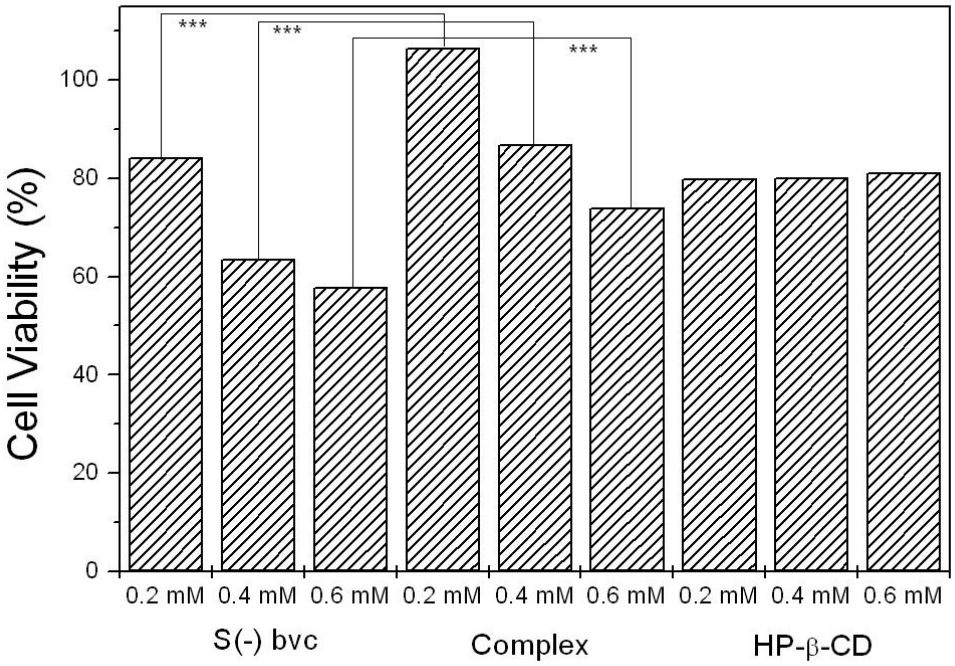

Figure 5. Cytotoxic effects of S(-) bvc, S(-) bvc:HP-β-CD, HP-β-CD at 0.2, 0.4 and 0.6 mM on Balb/c 3T3 cells incubated for 2 h at 37°C and 5% CO_2 as evaluated by MTT reduction test. Data expressed as % cell viability (Mean ± SD, n=8 experiments). *** $p<0.001$ (one-way ANOVA with Tukey-Kramer *post hoc* test).

been formed. Crystallinity was determined by comparing some representative peak heights in the diffraction patterns of the binary systems with those of a reference. The S(-) bvc diffractogram clearly reveals a crystalline (Figure 4d) nature while HP-β-cyclodextrin is amorphous (Figure 4a). The physical mixture of S(-) bvc and HP-β-CD gave superposed patterns of crystalline bupivacaine and the amorphous HP-β-CD (Figure 4c). On the other hand, the inclusion complex (Figure 4 b) produced a pattern in which the crystalline S(-) bvc has disappeared; in fact, the pattern in Figure 4b is similar to the diffractogram related to HP-β-CD, suggesting conformational changes for S(-) bvc in the inclusion complex. In the range 2θ=5-60, the diffractogram for the complex has a lower intensity than that for HP-β-CD (see Figure 4a and b), related to the reduced quantity of HP-β-CD. The reduction in the intensity was not 50%, compared to HP-β-CD, probably due to the contribution of the structural arrangement of the S(-) bvc inside HP-β-CD.

Varying the concentration of HP-β-CD had no effect on the cell viability. On the other hand, S(-) bvc reduced cell viability in a dose-dependent manner, down to 57% (0.6 mM S(-) bvc), while S(-) bvc: HP-β-CD induced a maximum inhibition (0.6 mM) comparable to that of HP-β-CD, i.e. affecting cell viability very little up to 2 hours after treatment ($p<0.001$), compared to S(-) bvc (Figure 5). One of the essential points about this in vitro toxicity model is that, since the cytotoxic effects of S(-) bvc are dose-dependent, the cellular protective effects observed on treatment with the S(-)bvc:HP-β-CD complex could be explained by the sustained release of S(-) bvc from the HP-β-CD cavity, as demonstrated by *in vitro* drug-release assays (Araujo, et al. 2004).

The physicochemical characterization and *in vitro* toxicity evaluation of an inclusion complex of S(-) bvc and HP-β-CD have been described. The complexation with HP-β-CD generated a less toxic S(-) bvc formulation. Therefore, we believe that this inclusion complex may be a potential therapeutic formulation for the treatment of pain. *In vivo* biological tests have been carried out with this formulation and will be published in due course.

ACKNOWLEDGMENTS

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and University of Sorocaba. CMM was the recipient of a fellowship from FAPESP (04/02091-4); EP is the recipient of a fellowship from CNPq. The authors would like to thank the X-Ray Laboratory at the Department of Physics of the Federal University of Paraná, Brazil.

RESUMO

Caracterização morfológica e estudo de citotoxicidade do complexo de inclusão entre S(-) bupivacaína e 2-hidroxipropil- β-ciclodextrina

Anestésicos locais são substâncias químicas que possuem

atividade farmacológica capaz de diminuir a dor devido a ligação destes em canais de sódio voltagem dependentes em membranas excitáveis, bloqueando a entrada de íons sódio e a propagação do impulso nervoso. A S(-) bupivacaína (S(-) bvc) é um anestésico local do tipo aminoamida muito utilizado clinicamente. Neste estudo foi realizada a caracterização morfológica e ensaios de citotoxicidade do complexo de inclusão entre S(-) bvc e 2-Hidroxipropil-β-ciclodextrina (HP-β-CD). Análises de calorimetria diferencial de varredura, microscopia eletrônica de varredura e Raio-X mostraram mudanças na morfologia do complexo de inclusão. Estudos iniciais de citotoxicidade mostraram que o complexo de inclusão diminui o efeito tóxico (p<0,001) da S(-) bvc. Estes resultados sugerem que o complexo de inclusão entre S(-)bvc e HP-β-CD representam uma alternativa promissora para o uso no tratamento da dor.

Palavras-chave: S(-) bupivacaína; ciclodextrina; complexo de inclusão.

REFERENCES

Araújo DR, Cereda CM, Brunetto GB, Pinto LMA, Santana MH, de Paula E. Encapsulation of mepivacaine prolongs the *analgesia provided by sciatic nerve blockade in mice. Can J Anaesth* 2004; 51:566-72.

Araújo DR, Fraceto LF, Braga AFA, de Paula E. Drug-delivery systems for racemic bupivacaine (S50-R50) and bupivacaine enantiomeric mixture (S75-R25):cyclodextrins complexation effects on sciatic nerve blockade in mice. *Rev Bras Anestesiol* 2005; 55:316-28.

Araújo DR, Moraes CM, Fraceto LF, Braga AFA, de Paula E. Cyclodextrin-bupivacaine enantiomeric mixture (S75-R25) inclusion complex and intrathecal anesthesia in rats. *Rev Bras Anestesiol* 2006; 56:495-506.

Bibby D, Davies NM, Tueker IG. Mechanisms by which cyclodextrins modify drug release from polymeric drug delivery systems. *Int J Pharm* 2000; 197:1-11.

Covino BG, Vassalo HG. *Local anesthetics*: mechanisms of action and clinical use. New York: Grune and Stratton; 1976. 255p.

Foster RH, Markham A. Levobupivacaine. A review of its pharmacology and use as a local anaesthetic. *Drugs* 2000; 59:551-79.

Grant GJ, Bansinath M. Liposomal delivery systems for local anesthetics. *Reg Anesth Pain Med* 2001; 26:61-3.

Gristwood RW. Cardiac and CNS toxicity of levobupivacaine: strengths of evidence for advantage over bupivacaine. *Drug Saf* 2002; 25:153-63.

Hirayama F, Uekama K. Cyclodextrin-based controlled drug release system. *Adv Drug Deliv Rev* 1999; 36:125-41.

Huang YF, Pryor ME, Mather LE, Veering BT. Cardiovascular and central nervous system effects of intravenous S-bupivacaine and bupivacaine in sheep. *Anesth Analg* 1998; 86:797-804.

Jong RH. *Local anesthetics*. Springfield: C.C. Thomas; 1994. 325p.

Kohata S, Jyodi K, Ohyoshi A. Thermal decomposition of cyclodextrins (α -, β -, γ, and modified β-CyD) and of metal-(β-CyD) complex in the solid phase. *Thermochim Acta* 1993; 217:187-98.

Loftsson T, Brewster ME. Pharmaceutical application of Cyclodextrin. 1. Drug solubilization and stabilization. *J Pharm Sci* 1996, 85:1017-25.

Loukas YL, Vraka V, Gregoriadis G. Novel non-acidic formulations of haloperidol complexed with beta-cyclodextrin derivatives. *J Pharm Biomed Anal* 1997; 16:263-8.

Mather LE, McCall P, McNicol PL. Bupivacaine enantiomer pharmacokinetics after intercostal neural blockade in liver transplant patients. *Anesth Analg* 1995; 80:328-35.

Michaud M, Icart S. Determination of the substitution of hydroxypropylbetadex using fourier transform infrared spectrophotometry. *PharmEuropa* , 2001; 13:714-6.

Naidu NB, Chowdary KPR, Murthy KVR, Satyanarayana V, Hayman AR, Becket G. Physicochemical characterization and dissolution properties of meloxicam-cyclodextrin binary systems. *J Pharm Biomed Anal* 2004; 35:75-86.

Pinto LMA, Fraceto LF, Santana MHA, Pertinhez TA, Oyama S, de Paula E. Physico-chemical characterization of benzocaine-β-cyclodextrin inclusion complexes. *J Pharm Biomed Anal* 2005; 39:956-63.

Ren X, Xue Y, Liu J, Zhang K, Zheng J, Lou G, Gou C, Mu Y, Shen J. A novel cyclodextrin-derived tellurium compound with glutathione peroxidase. *Chembiochem* 2002; 3:363-5.

Rose JS, Neal JM, Kopacz DJ. Extended-duration analgesia: update on microspheres and liposomes. *Reg Anesth Pain Med* 2005; 30:275-85.

Strichartz GR, Ritchie JM. *Local anesthetics*: handbook of experimental pharmacology. Berlin: Springer-Verlag; 1987. 445p.

Thompson D.O. Cyclodextrin-enabling excipients: their present and future use in pharmaceuticals. *Crit Rev Ther Drug Carrier Syst* 1997; 14:1-104.