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International Journal of Pharmaceutics 331 (2007) 99–106
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Study of the interaction between *S*(−) bupivacaine and 2-hydroxypropyl-β-cyclodextrin

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Received 8 February 2006; received in revised form 20 September 2006; accepted 21 September 2006
Available online 5 October 2006

Abstract

Local anesthetics are substances able to induce pain relief by binding to the sodium channel of excitable membranes, blocking the influx of sodium ions and the propagation of nervous impulses. *S*(−) bupivacaine (*S*(−) bvc) is an amide type local anesthetic widely used in surgery and obstetrics for sustained peripheral and central nerve blockade. The present work focuses on the characterization of an inclusion complex of *S*(−) bvc in 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). The complexation with HP-β-CD has been investigated using reversed-phase liquid chromatography and solubility isotherms. The retention behavior was analyzed on a reversed phase C_{18} column and the mobile phase used was acetonitrile–phosphate buffer pH 7.4 (10 mM), (45/55, v/v), in which HP-β-CD was incorporated as a mobile phase additive. The decrease in the retention times with increasing concentration of HP-β-CD enables the determination of the complex apparent stability constants by HPLC as a function of temperature. The solubility isotherms were studied as a function of pH (7.4 and 10.5) and temperature. The pH study showed that *S*(−) bvc reaches a concentration at least 1.5 and 4.5 times higher (pH 7.4 and 10.5, respectively) than the one presented by the free drug in water. The calculated values for the apparent stability constant (*K*) are 13.1 ± 2.8 and $95.4 \pm 11.8\ M^{-1}$ for pH 7.4 and 10.5, respectively, thus indicating the formation of a stable complex. In addition, the study of the apparent stability constant by HPLC and solubility isotherm gives thermodynamics information about the interaction between *S*(−) bvc and HP-β-CD. The application of the continuous variation method indicated the presence of a complex with 1:1 *S*(−) bvc:HP-β-CD stoichiometry. This is an important study for the characterization of potential formulations to be used as therapeutic options for the treatment of pain.

Keywords: *S*(−) Bupivacaine; 2-Hydroxypropyl-β-cyclodextrin; HPLC; Solubility isotherm; Nuclear magnetic resonance

1. Introduction

Local anesthetic (LA) works along the axons, blocking the action potential (Covino and Vassalo, 1976; Ritchie and Strichartz, 1987; de Jong, 1994). At the present time, there is a strong clinical need for long-acting LA as well as molecules with decreased systemic uptake, what leads to less toxic side effects. Bupivacaine is an amide type local anesthetic widely used in surgery and obstetrics for sustained peripheral and central nerve blockade. Its structure is formed by a single chiral centre and is marketed as the racemate of *R*(−) and *S*(−) bupivacaine (rac-bupivacaine). Both enantiomers are active, however, a longer duration of neural blockade as well as lower propensity towards central nervous system and cardiovascular toxicity are produced by *S*(−) bupivacaine (Fig. 1) (Mather et al., 1995; Huang et al., 1998; Gristwood, 2002). This fact has led to the introduction of *S*(−) bupivacaine into clinical practice under the name of levobupivacaine (Foster and Markham, 2000). The compound safety and efficacy have been compared with that of rac-bupivacaine in surgical anesthesia and pain management (Foster and Markham, 2000).

The development of local anesthetic formulations in carriers such as liposomes, glucose polymers, dextran, hyaluronic

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doi:10.1016/j.ijpharm.2006.09.054

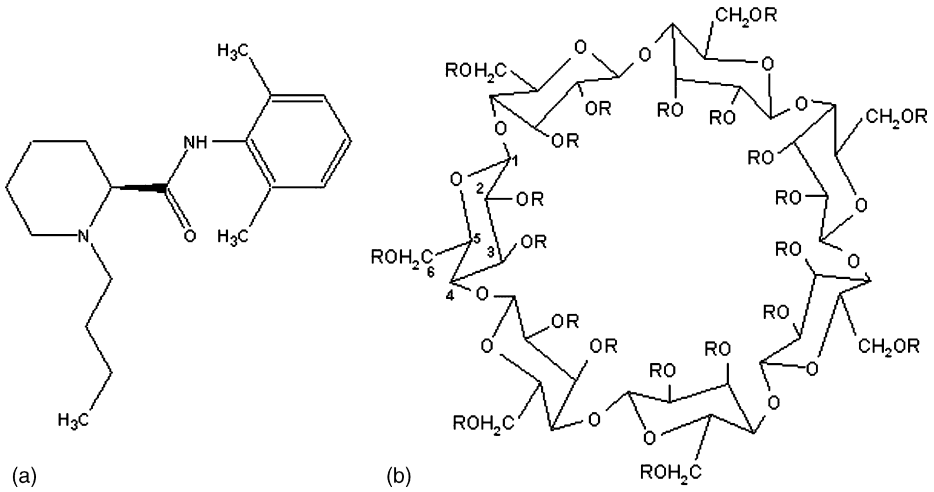

Fig. 1. Chemical structure of (a) *S*(−) bvc and (b) HP-β-CD (R = isopropyl group)—schematic representation.

acid, biopolymers, microspheres, among others could offer the possibility of controlling drug delivery in biological systems, prolonging the anesthetic effect and reducing its toxicity (Ellis et al., 1995; Kern and Langevin, 2000). Complexation with cyclodextrins (CD) provides a way to increase the solubility, stability and bioavailability of drugs (Karim et al., 2001; Dollo et al., 1998). CD is able to form inclusion complexes with different classes of molecules, modifying their physical, chemical and biological properties (Rajewski and Stella, 1996). These cyclic polymers are formed by glucose molecules linked through 1–4 bonds and can be composed by 6 (α-CD), 7 (β-CD) or 8 (γ-CD) glucose units. β-Cyclodextrin (Fig. 1, R = H) has been extensively studied despite its low aqueous solubility. Moreover, its alkylated derivatives, e.g. 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), have attracted growing interest due to their improved complexing ability, greater water solubility and lesser toxicity. The non-polar cavity of CDs can form inclusion compounds with a variety of guest molecules, being the binding governed by the molecular polarity and ability to closely fit within the cavity (Connors, 1997). Furthermore, the CD allows the accommodation of the apolar part of some molecules such as local anesthetics. Some other studies about the complexation of LA molecules with CD have been reported in the literature (Freville et al., 1996; Dollo et al., 1996a,b, 1998, 2000).

Two approaches are available for applying CDs in reversed-phase HPLC: either CDs can be in the stationary phase, chemically bound to silica gel, or they can be used as mobile phase additives. As a result of host–guest interactions, the retention time of the guest will change; it will be shorter when complexation occurs in the mobile phase and longer when it takes place in the stationary phase. These changes in the retention behavior are closely related to the stability constants of the complexes formed. The determination of apparent stability constants of different inclusion compounds has been previously reported (Sadlej-Sosnowska, 1995; Tang and Love, 1997; Morin et al., 1998a,b; Bielejewska et al., 2001).

The aim of the present study was to characterize some inclusion complexes through the study of the HPLC retention behavior of *S*(−) bupivacaine (*S*(−) bvc) in presence of HP-β-CD and to estimate the stability constants as a function of temperature. The complex thermodynamic parameters are going to give further information about its enthalpy and entropy. This is an elementary study for the characterization of a potential formulation to be used as a therapeutic option for the treatment of pain (de Araújo et al., 2003, 2005).

2. Experimental

2.1. 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 by a substitution degree of 4.2 based on a Fourier transformed infrared spectrophotometry (FT-IR) method (Michaud and Icart, 2001). HPLC-grade acetonitrile (ACN) was obtained from J.T. Baker. Sodium dihydrogen phosphate and disodium hydrogen phosphate (anhydrous) were from Sigma Chem. Co. and deionized water at 18 mΩ from a Waters ultra pure water system.

2.2. Effect of cyclodextrin in S(−) bvc retention time by HPLC

The chromatographic experiments were performed using a Shimadzu SCL-10VP controller pump, a Shimadzu SIL-10AD VP auto injector, a UV–vis SPD-10A VP detector (detection: 220 nm for *S*(−) bupivacaine) and Class-VP 6.12 as software. A reversed phase Phenomonex Gemini C_{18}, 5 μm, 10 cm × 0.46 cm was employed. The mobile phase used for these studies was acetonitrile–phosphate buffer pH 7.4 (10 mM), (45/55, v/v), in which HP-β-CD was dissolved (5, 10, 15, 20, 25 mM). The whole solution was filtered through a 0.2 μm pore size nylon membrane filter. The mobile phase was pumped at a flow rate of 1.5 mL/min. The chromatographic experiments were carried out at a range of temperatures (293–311 K). The *S*(−) bvc concentration in the injected solution was 1 mM and the injection volume was 0.2 mL in all experiments.

The retention behavior of *S*(−) bvc is governed by the drug partition coefficients between the mobile and stationary phases. In the presence of cyclodextrins, there is an additional contribution in the drug retention behavior due to the complexation process.

The capacity factors for *S*(−) bvc were monitored in the presence of increasing concentration of HP-β-CD. The apparent stability constant of the complex, *K*, was determined in triplicate, using the following equation (Atwood et al., 1996):

$$\frac{1}{k'} = \frac{1}{k'_s} + \frac{K[\mathrm{CD}]^x}{k'_s} \quad (1)$$

where k' is the capacity factor at each cyclodextrin concentration [CD], and k'_s is the solute capacity factor in absence of cyclodextrin. For a 1:1 stoichiometry complex, a plot of $1/k'$ versus [CD] yields a straight line and *K* is obtained from the slope-to-intercept ratio.

2.3. Determination of the apparent stability constants by solubility studies

2.3.1. The pH effect

As *S*(−) bvc is a weak base (pK_a 8.1), the pH effects on the solubility were carried out in pH 10.5 (carbonate buffer, 20 mM) and pH 7.4 (phosphate buffer, 20 mM) at 25 °C. Excess amounts of *S*(−) bvc were added to 10 mL glass tubes containing different concentrations of HP-β-CD. The tubes were shaken until equilibrium was reached (24 h). Afterward, the solutions were centrifuged and the concentration of *S*(−) bvc was spectrophotometrically determined at 220 nm using a Femto spectrophotometer. The presence of HP-β-CD did not interfere in the spectrophotometric assay of *S*(−) bvc.

When a linear relationship between the solubility of *S*(−) bvc and the concentration of HP-β-CD is obtained, the diagram is classified as A_L, according to Higuchi and Connors (1965) and the experimental data fit Eq. (2):

$$K = \frac{\text{slope}}{S_o(1 - \text{slope})} \quad (2)$$

where S_o is the *S*(−) bvc molar solubility. The apparent stability constant of the complex formed, *K*, can be obtained from the slope of the straight line.

2.3.2. The temperature effect

Excess amounts of *S*(−) bvc were added to 10 mL glass tubes containing different concentrations of HP-β-CD (carbonate buffer, 20 mM, pH 10.5). The tubes were shaken until equilibrium was reached (24 h) at variable temperature (10–40 °C). Afterward, the solutions were centrifuged and the concentration of *S*(−) bvc was spectrophotometrically determined at 220 nm using a Femto spectrophotometer. The presence of HP-β-CD did not interfere in the spectrophotometric assay of *S*(−) bvc. After that, the apparent stability constant of the complex formed, *K*, could be obtained from the slope of the straight line (Eq. (2)).

2.4. Thermodynamic relationship

The ΔH° and ΔS° represent, respectively, the standard enthalpy and entropy of the *S*(−) bvc transference from the mobile phase to the cyclodextrin cavity. These energies can be calculated using the following thermodynamic relationships (Eq. (3)) (Ismaili et al., 2003; Ravelet et al., 2002a,b; Rozou et al., 2004):

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where *T* is the temperature and *R* is the gas constant. For linear van't Hoff plot (ln *K* versus $1/T$), the slope and the intercept are, respectively, $-\Delta H^\circ/R$ and $\Delta S^\circ/R$.

2.5. Stoichiometry of the complex by nuclear magnetic resonance

The continuous variation method was adopted to determine the stoichiometry of the complex (Djedaïne et al., 1990). ^{1}H NMR spectra were obtained for a series of *S*(−) bvc:HP-β-CD mixtures in which the total initial concentration of both species was kept constant (5 mM) but the mole fraction of each component varied from 0 to 1.

One-dimensional ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz spectrometer, in unbuffered deuterated water (D_2O) for *S*(−) bvc/HP-β-CD system. The stock solutions of *S*(−) bvc (5 mM) and HP-β-CD (5 mM) were mixed in 5 mm NMR tubes, giving a total sample volume of 600 μL, and left overnight for equilibration before the NMR analysis. The probe temperature was regulated to 298 K.

The ^{1}H NMR spectra were recorded using a simple pulse-acquire sequence with solvent presaturation. Typical acquisition parameters consisted of 32 K points covering a sweep width of 6000 Hz, a pulse width of 10 μs, digital zero filling to 128 K and a 0.5 Hz exponential function were applied to the FID before Fourier transformation. The resonance at 4.81 ppm due to residual solvent, present as impurities (H_2O and HDO), was used as internal reference. The data were collected without an external reference to avoid possible interactions with the HP-β-CD.

3. Results and discussion

3.1. Chromatographic determination of the apparent stability constants

When cyclodextrins are added to the mobile phase, solute retention is driven by the drug partition between the mobile and stationary phases and the solute complexation with cyclodextrins. According to the solute retention time and the void time, capacity factors were calculated for each solute in the presence of increasing concentrations of HP-β-CD. As expected, the retention times decrease as the concentration of HP-β-CD in the mobile phase increases due to the formation of the analyte–cyclodextrin complex, which enhances the guest

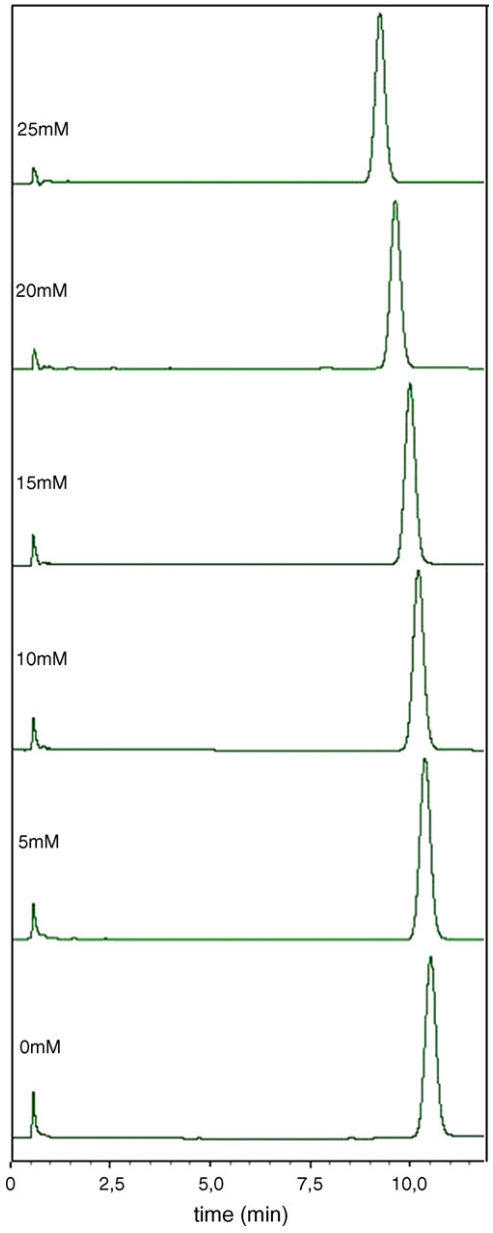

Fig. 2. Decrease in *S*(−) bvc retention time in the presence of increasing concentrations of HP-β-CD (0, 5, 10, 15, 20, 25 mM) at 26 °C. Chromatographic conditions—column: Phenomenex C18, 5 μm, 10 cm × 0.46 cm; mobile phase: acetonitrile/phosphate buffer pH 7.4, 10 mM (45/55, v/v).

solubility in the mobile phase and reduces its residence time in the column (Uekama et al., 1978a,b); an assay is shown as an example in Fig. 2. The variation in the retention times obtained by replication was always lower than 1%.

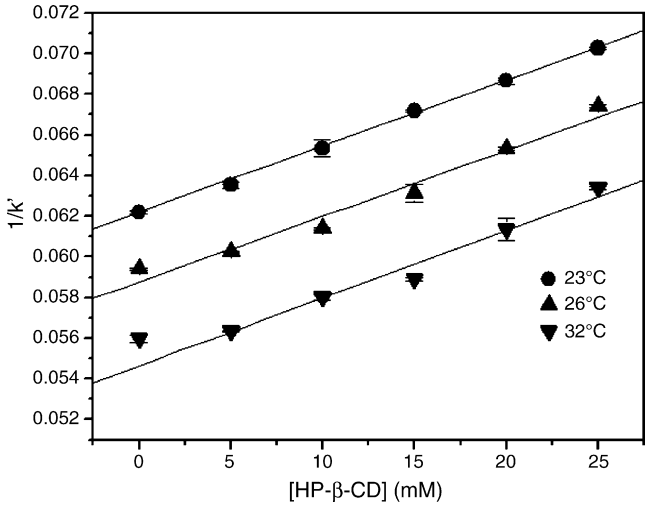

Fig. 3. Plot of 1/*k* vs. HP-β-CD concentration for *S*(−) bvc in (●) 23 °C, (▲) 26 °C and (▼) 32 °C. Chromatographic conditions as in Fig. 2.

The above mentioned formation constant for the *S*(−) bvc–HP-β-CD complex was calculated according to several temperatures. The linear relationship between 1/*k*′ and HP-β-CD concentration (Fig. 3) with correlation coefficients higher than 0.99 indicates that the behavior of *S*(−) bvc is well described by the model, assuming a 1:1 stoichiometry between the guest and HP-β-CD as a function of temperature (Ravelet et al., 2002a,b).

The apparent stability constants obtained are shown in Table 1. These values are slightly lower than those determined by spectrophotometric measurements for complexation of bvc (racemic) with HP-β-CD at pH 7.4 and 25 °C (de Araújo et al., 2005).

The amount of acetonitrile present in the mobile phase and the time needed by the complex formation to reach equilibrium could explain the differences detected. It is well known that the addition of acetonitrile or methanol may have a negative effect on complex formation with cyclodextrin. For these studied systems, it seems that the addition of organic solvents led to a decrease in the apparent binding constants. There are several factors which may contribute to this decrease; firstly, the amount of organic solvent results in a less polar mobile phase in which the non-polar solutes become more soluble and as a consequence, the solute affinity for the hydrophobic cavity of HP-β-CD diminishes and part of the driving force for inclusion is removed. Secondly, a phenomenon of competition between the solute and the organic solvent for binding HP-β-CD may occur, even though organic solvent binds weakly to HP-β-CD (Gazpio et al., 2004).

Table 1
Values of apparent stability constant from the *S*(−) bvc:HP-β-CD complex obtained from the HPLC experiments (Fig. 3)

T (K)	1/*T*	*K* (M^{-1})	ln *K*	Slope ($\times 10^{-4}$)	Intercept	Correlation coefficient, *r*
293	0.003413	4.95 ± 0.31	1.59	3.066	0.06199	0.994
296	0.003378	5.22 ± 0.02	1.65	3.246	0.06221	0.999
299	0.003344	5.51 ± 0.05	1.70	3.237	0.05876	0.996
305	0.003279	6.10 ± 0.32	1.380	3.330	0.05464	0.988
311	0.003215	6.98 ± 0.40	1.94	3.709	0.05314	0.992

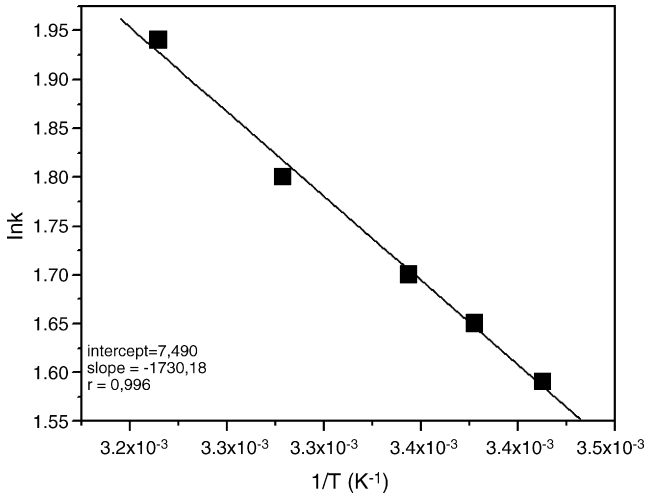

Fig. 4. van't Hoff plots (ln K vs. $1/T$) for $S(-)$ bvc:HP-β-CD associations determined by HPLC experiments.

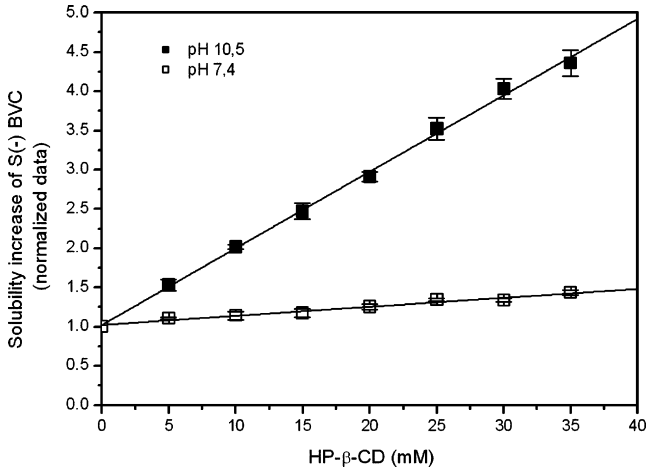

Fig. 5. Solubility diagrams of $S(-)$ bvc with HP-β-CD in different pH (7.4 and 10.5) at room temperature. Absorbances are normalized.

As can be observed in Table 1, the apparent stability constants of the HP-β-CD complex with $S(-)$ bvc show an increase when the temperature rises. In order to gain information about the mechanistic aspect of the HP-β-CD affinity for $S(-)$ bvc, the thermodynamic parameters were obtained from van't Hoff plot. The ln K versus $1/T$ plots were obtained from Table 1. Data are shown in Fig. 4. Linear van't Hoff plot was obtained with a correlation coefficient of 0.996.

Fig. 4 shows that from the van't Hoff plot it was determined ΔH° and ΔS° for the HP-β-CD and $S(-)$ bvc complexation, being 14.37 kJ/mol and 62.24 J/mol K values, respectively. From these values, the Gibbs free energy calculated is −4.17 kJ/mol.

3.2. pH effect on the solubility studies

The solubility enhancements obtained with cyclodextrins have been widely employed in the improvement of drugs bioavailability (Hirayama and Uekama, 1999; Tommasini et al., 2004). The $S(-)$ bvc is a local anesthetic that presents a pK_a 8.1. Consequently, to verify the pH effect on its interaction with the HP-β-CD cavity, the solubility isotherms are determined in pH 7.4 (phosphate buffer) and 10.5 (carbonate/bicarbonate buffer). In both pH, the increase in solubility occurred as a linear function of HP-β-CD concentration (Fig. 5), corresponding to the A_L-type profile defined by Higuchi and Connors (1965). This relationship suggests the formation of a 1:1 $S(-)$ bvc:HP-β-CD complex. The apparent stability constant (K) determined from the slope and the intercept of these plots are 13.1 ± 2.8 and $95.4 \pm 11.8\ M^{-1}$ for pH 7.4 and 10.5, respectively, thus indicating the formation of a stable complex (Loukas et al., 1997).

The differences in the results obtained from these data reveal the importance of the pH on adequate fitting between host and guest molecules. The values obtained from the apparent stability constant are in agreement with similar reports in the literature (Dollo et al., 1998; de Araújo et al., 2005).

In fact, the results herein obtained demonstrate that in the presence of 35 mM HP-β-CD, $S(-)$ bvc reaches a concentration at least 1.5 times higher (pH 7.4) and 4.5 times higher (pH 10.5) than those of the free drug in water (Fig. 5). The results are summarized in Table 2.

The solubility increase caused by complexation of $S(-)$ bvc with HP-β-CD is lower than reported for others drugs in literature (Loftsson and Brewster, 1996). *In vivo* experiments for a similar system with racemic bvc and cyclodextrin produce an increase in duration and intensity of local anesthesia (de Araújo et al., 2005, 2006).

3.3. The temperature effect on solubility studies

The temperature effect in the solubility isotherm for complex formation between $S(-)$ bvc and HP-β-CD is shown in Fig. 6. The apparent stability constant (K) determined from the slope and the intercept of the temperature isotherms is shown in Table 3.

As determined by HPLC method, the apparent stability constants increase when the temperature rises. The thermodynamic parameters were calculated by van't Hoff plot. Data are shown in Fig. 7. Linear van't Hoff plot was obtained with a correlation coefficient of 0.997.

From Fig. 8 data, ΔH° and ΔS° values determined for the HP-β-CD and $S(-)$ bvc complexation are 4.75 kJ/mol and

Table 2
Apparent stability constants, K, for $S(-)$ bvc:HP-β-CD inclusion complexes in pH 7.4 (phosphate buffer, 20 mM) and 10.5 (carbonate buffer, 20 mM) determined by phase solubility techniques

pH	Correlation coefficient, r	Slope	Intercept, S_o ($\times 10^{-3}$)	Apparent stability constant, K
7.4	0.993	0.036 ± 0.002	3.29 ± 0.5	13.1 ± 2.8
10.5	0.998	0.013 ± 0.001	0.137 ± 0.005	96.14 ± 11.8

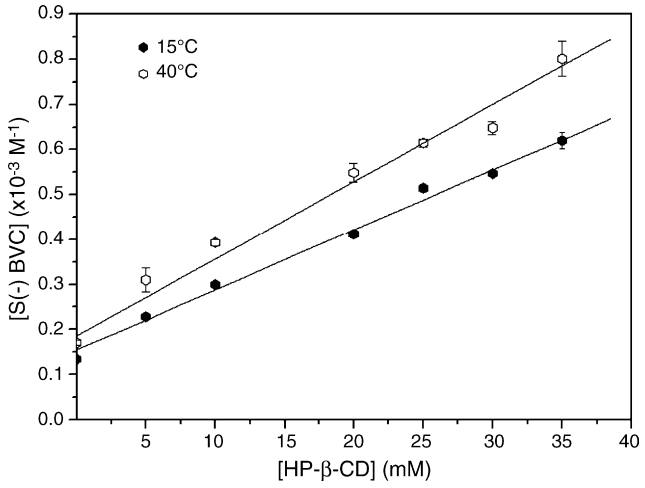

Fig. 6. Solubility diagrams of *S*(−) bvc with HP-β-CD in different temperature (15 and 40 °C) at pH 10.5.

Table 3
Apparent stability constants, *K*, for *S*(−) bvc:HP-β-CD inclusion complexes in pH 10.5 (carbonate buffer, 20 mM) determined by phase solubility techniques

T (K)	*K* (M^{-1})	Slope ($\times10^{-5}$)	Intercept ($\times10^{-4}$)	Correlation coefficient, *r*
283	83.0 ± 1.02	1.395	1.776	0.979
288	86.4 ± 1.28	1.333	1.545	0.995
293	90.0 ± 1.22	1.395	1.315	0.997
303	95.5 ± 1.03	1.460	1.517	0.998
313	100.4 ± 1.03	1.716	1.851	0.991

53.48 J/mol K, respectively. From these values, the Gibbs free energy calculated is −11.19 kJ/mol.

3.4. Thermodynamic analysis

The formation of an inclusion complex with cyclodextrin is classically attributed to interactions with ΔH° negative values for inclusion complexation with guest and cyclodextrins, arising from the stronger hydrophobic interaction, whereas the ΔS° values are either negative or slightly positive, indicating inclusion complexation with guest without accompanying extensive desolvation, indicating that these inclusion reactions are primarily enthalpy-driven processes (Stalcup et al., 1990; Tong et al., 1991; Morin et al., 1998a,b; Flood et al., 2000; Ismaili et al., 2003; Martin Del Valle, 2004).

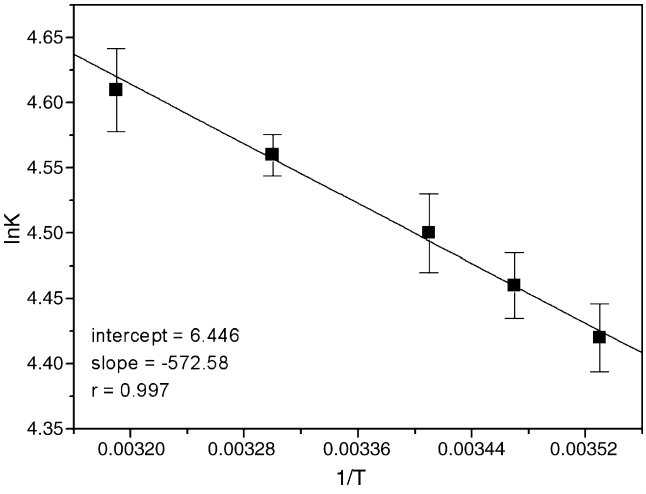

Fig. 7. van't Hoff plots (ln *K* vs. 1/*T*) for *S*(−) bvc:HP-β-CD associations, determined by solubility diagrams experiments.

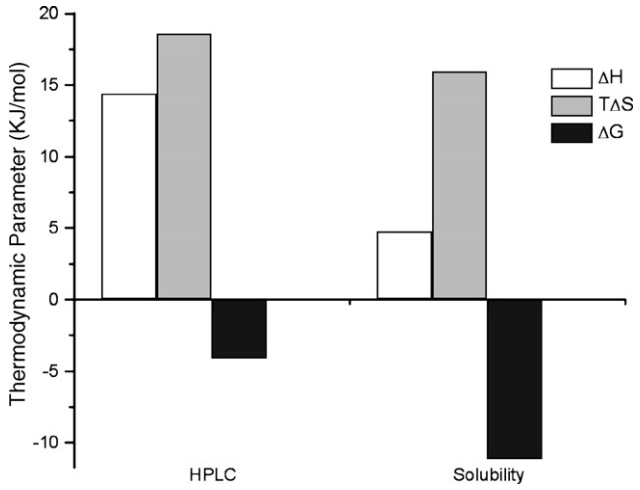

Fig. 8. Free energy (ΔG°), enthalpy (ΔH°) and entropy changes ($T\,\Delta S^{\circ}$) for the inclusion complexation of *S*(−) bvc with HP-β-CD determined by HPLC and solubility isotherm at pH 25 °C.

However, for *S*(−) bvc guest molecule with HP-β-CD different results were obtained. Apparently, when the *S*(−) bvc molecule is free in solution, it seems to present a strong interaction with the solvent due to a shell surrounding the molecule. Upon binding, this solvent shell is broken up, leading to the partly unfavorable enthalpy change (ΔH). Furthermore, the inclusion complexation demands fairly extensive desolvation of both *S*(−) bvc and HP-β-CD, affording the highly positive entropy change upon complexation as observed in Fig. 8. On the other hand, the inclusion complexation of *S*(−) bvc with HP-β-CD gave entirely positive enthalpy change (ΔH), indicating that these inclusion reaction for *S*(−) bvc are mainly entropic-driven processes. Its suggests that HP-β-CD form a suitable conformation, favorable to the complexation with *S*(−) bvc from the viewpoint of entropy, but unfavorable to the complexation from the adjusted induced-fit concept, giving lower stability constant. Similar results are described in literature (Liu et al., 2002).

3.5. Stoichiometry of the complex

The continuous variation method was employed to establish the stoichiometry of the complex using NMR. The assignments of ^{1}H-chemical shift of *S*(−) bvc (Fraceto et al., 2005) and HP-β-CD (Xiliang et al., 2005) are according to the literature. The *S*(−) bvc induced ^{1}H-chemical shifts changes in HP-β-CD hydrogens, mainly in H_3 and H_5 hydrogens (data not shown).

If a physical parameter directly related to the concentration of the complex is plotted as a function of the mole fraction (*r*) of *S*(−) bvc or HP-β-CD, its maximal value will occur at

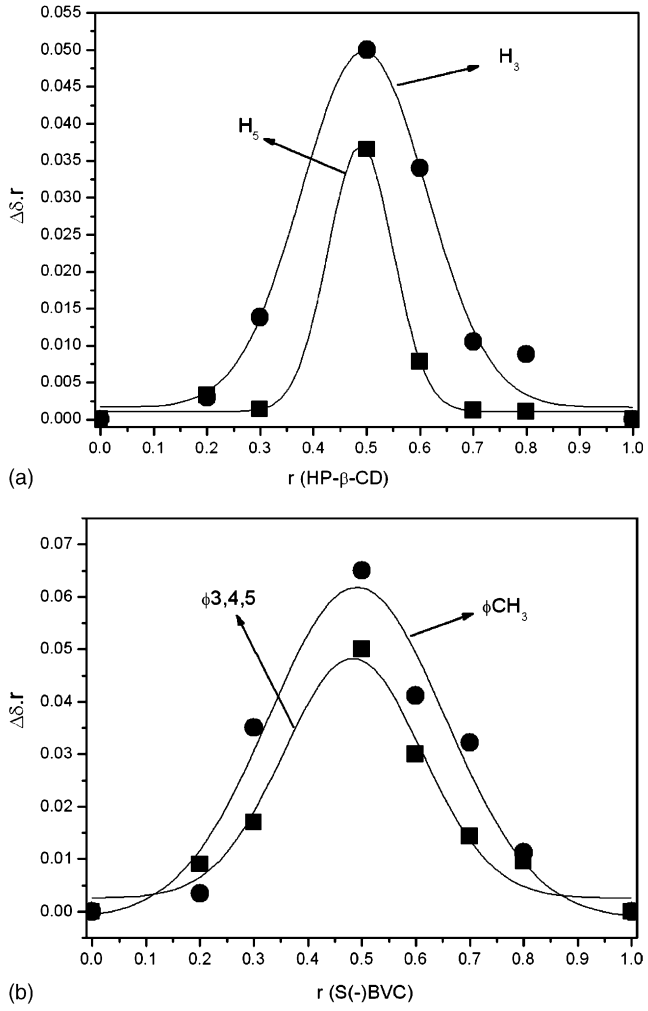

Fig. 9. Job's plots corresponding to the chemical shift displacement of (a) H_3 and H_5 hydrogens for HP-β-CD and (b) aromatic and ortoaromatic methyl groups for *S*(−) bvc.

$r_{S(-)\text{bvc}} = m/(m+n)$ or $r_{\text{HP-β-CD}} = n/(m+n)$, where m and n are the molar ratios of *S*(−) bvc and HP-β-CD in the complex, respectively. In the NMR, and under fast conditions, for a signal belonging to *S*(−) bvc, for example, the calculated quantity $\Delta\delta$. [*S*(−) bvc] will be proportional to the complex concentration and, can thus be plotted against *r* (Djedaïne et al., 1990).

The continuous variation method was applied for all hydrogens of the molecules (host and guest) and yielded identical results. For the sake of conciseness, only the *S*(−) bvc and HP-β-CD that experienced the largest shifts have been reported in Fig. 9.

In all cases, Job's plots show a maximum value at $r = 0.5$ and highly symmetrical shape, indicating the existence of complex with a 1:1 stoichiometry, within the range of the investigated concentrations. This results are in agreement with the phase-solubility studies between *S*(−) bvc and HP-β-CD.

This study provides perspectives for future experiments using this inclusion complex with HP-β-CD in order to verify its therapeutic efficacy. *In vitro* and *in vivo* tests are being carried out with this formulation and will be published in due time.

Acknowledgments

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo. C.M.M. is the recipient of fellowship from FAPESP (04/02091-4); P.A. is the recipient of fellowship from PROBIC-UNISO; E.P. is the recipient of a fellowship from CNPq.

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