Hydroxymethylnitrofurazone:Dimethyl- β -cyclodextrin Inclusion Complex: A Physical–Chemistry Characterization

Renato Grillo · Nathalie Ferreira Silva Melo ·
Carolina Morales Moraes · André Henrique Rosa ·
José Arnaldo Frutuoso Roveda · Carla M. S. Menezes ·
Elizabeth Igne Ferreira · Leonardo Fernandes Fraceto

Received: 13 September 2007 / Accepted: 3 January 2008 /

Published online: 27 February 2008

© Springer Science + Business Media B.V. 2008

Abstract Hydroxymethylnitrofurazone (NFOH) is active against *Trypanosoma cruzi*; however, its low solubility and high toxicity precludes its current use in treatment of parasitosis. Cyclodextrin can be used as a drug carrier system, as it is able to form inclusion (host–guest) complexes with a wide variety of organic (guest) molecules. Several reports have shown the interesting use of modified β -cyclodextrins in pharmaceutical formulation, to improve the bioavailability of drugs and to decrease their toxicity. The aim of this work was to characterize inclusion complexes formed between NFOH and dimethyl- β -cyclodextrin (DM- β -CD) by complexation/release kinetics and solubility isotherm experiments using ultraviolet (UV)-visible spectrophotometry and by the measurement of the dynamics information obtained from T_1 relaxation times and diffusion (DOSY) experiments using nuclear magnetic resonance (NMR) spectroscopy. The complex was prepared at different NFOH and DM- β -CD molar ratios. The UV-visible measurements were recorded in a spectrophotometer, and NMR experiments were recorded at 20°C on a NMR spectrometer (Varian Inova) operating at 500 MHz. Longitudinal relaxation times were obtained by the conventional inversion-recovery method and the DOSY experiments were carried out using the BPPSTE sequence. The kinetics of complexation revealed that 30 h is enough for stabilization of the NFOH absorbance in presence of cyclodextrin. Solubility isotherm studies show a favorable complexation and increase in solubility when NFOH interacts with cyclodextrin. The analysis of the NMR-derived diffusion coefficients and T_1 relaxation times shows that in the presence of DM- β -CD, NFOH decreases its mobility in solution, indicating that this antichagasic compound interacts with the cyclodextrin cavity.

R. Grillo · N. F. S. Melo · A. H. Rosa · J. A. F. Roveda · L. F. Fraceto (✉)

Department of Environmental Engineering, State University of São Paulo, Campus Sorocaba,
Av. Três de Março, 511, CEP 18087-180 Sorocaba, São Paulo, Brazil
e-mail: leonardo@sorocaba.unesp.br

C. M. Moraes · L. F. Fraceto

Department of Biochemistry, Institute of Biology, State University of Campinas, Campinas, SP, Brazil

C. M. S. Menezes · E. I. Ferreira

LAPEN, Faculty of Pharmaceutical Science, University of São Paulo, São Paulo, SP, Brazil

The release kinetics assays showed that NFOH changes its release profile when in the presence of cyclodextrin due to complexation. This study was focused on the physicochemical characterization of drug-delivery formulations that may serve as potentially new therapeutic options for the treatment of Chagas' disease.

Keywords Hydroxymethylnitrofurazone · Dimethyl- β -cyclodextrin · Solubility isotherm · Nuclear magnetic resonance

1 Introduction

Chagas' disease is an important social and medical ailment for people living in the Americas. It is endemic in 21 countries, with approximately 16–18 million individuals infected with *Trypanosoma cruzi* and about 100 million people at risk of contracting the parasitosis, including those living in Northern hemisphere regions that received intense migratory currents from Ibero-American countries. Current therapy is based on nifurtimox and benznidazol, which are mostly effective in the acute phase of the disease and may cause serious adverse side effects. In Brazil, the only drug available on the market is benznidazol, in spite of the fact that Brazilian *T. cruzi* strains are more resistant to this drug than those from other countries in South America. Thus, new and better drugs are urgently needed to face this serious situation. The driving force for antichagasic drug development has been the selectivity of action, through the knowledge of biochemical targets in the parasite [1]. Hydroxymethylnitrofurazone (NFOH, Fig. 1) is one of the new candidate drugs for Chagas'

Fig. 1 Chemical structure of **a** NFOH and **b** DM- β -CD—(schematic representation)

disease chemotherapy that showed to be, *in vitro*, very potent against *T. cruzi* [1]. However, NFOH presents low aqueous solubility (ca 1.3 mM), high photodecomposition, and high toxicity.

Cyclodextrins (CD), which have a truncated cone shape, are able to form inclusion complexes with different classes of molecules, modifying their physical, chemical, and biological properties [2]. These cyclic polymers are formed by glucose molecules linked through one to four bonds and can be composed of 6 (α -CD), 7 (β -CD), or 8 (γ -CD) glucose units. β -cyclodextrin (Fig. 1, R=H) has been extensively studied despite its very low aqueous solubility when compared to other cyclodextrins. Moreover, modified cyclodextrin derivatives have attracted growing interest due to their improved complexing ability, greater water solubility, and lesser toxicity. The nonpolar cavity of CDs can form inclusion compounds with a variety of guest molecules, with the binding governed by the molecular polarity and ability to closely fit within the cavity [3]. Furthermore, CD allows the accommodation of the apolar part of some molecules. Complexation is determined by hydrophobic interactions, hydrogen bonds, van der Waals interactions, conformational energy, dipole–dipole and ion–dipole interactions, and the rearrangement of water molecules originally surrounding both cyclodextrin and the guest molecule.

In the present paper, we characterize inclusion complexes formed between NFOH and dimethyl- β -cyclodextrin (DM- β -CD) by complexation/release kinetics and solubility isotherm experiments using ultraviolet (UV)-visible spectrophotometry and by the measurement of dynamics information obtained from T_1 relaxation times and diffusion (DOSY) experiments using nuclear magnetic resonance (NMR) spectroscopy.

2 Materials and Methods

2.1 Reagents and Chemicals

Hydroxymethylnitrofurazone was synthesized as previously described [1]; DM- β -CD (degree of substitution~1.7–1.9) was obtained as a gift from the Roquette Company (Lestren, France). The solutions were prepared with ultrapure deionized water with maximum resistivity up to 18.2 megohms (or conductivity of 0.055 μ S, Waters ultrapure water system). The solutions were filtered through 0.22 μ m Millipore[®] nylon membrane filters (Belford, USA). The deuterated solvent was obtained from Sigma Chemicals.

2.2 Solid Inclusion Complex Preparation

The inclusion complex was prepared by shaking appropriate amounts of NFOH and DM- β -CD (1:1 molar ratio) in deionized water at room temperature ($25\pm1^\circ\text{C}$) for 20 h. After reaching equilibrium, the solution was freeze-dried (Labconco-freeze dry system/Freezone[®] 4.5) and stored at -20°C for further use.

2.3 Kinetics of Complexation and Phase Solubility Studies

The kinetics of complex formation was determined by shaking appropriate amounts of NFOH (0.2 μ M) and DM- β -CD in deionized water at room temperature ($25\pm1^\circ\text{C}$). The absorbance of NFOH was measured against the time during a period of 118 h (λ_{260} nm). The absorbance values were normalized and plotted against time to observe the time needed by the complexes to reach equilibrium in solution. For the phase solubility assays, an

excess of NFOH (5 mM) was mixed with aqueous solution with increased DM- β -CD concentrations (0–70 mM). The samples were stirred, and an aliquot was filtered through a 0.22- μ m membrane filter (Millipore). The amount of soluble NFOH was spectrophotometrically determined at 260 nm. The association constant (K_a) was determined from the slope of the linear portion of the phase-solubility diagram, according to (1) [4]:

$$K_a = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

where S_0 is the aqueous solubility of NFOH. All the experiments were carried out in triplicate.

Then, it was possible to calculate the complexation efficiency (CE) and the drug: cyclodextrin ratio (D:CD), according to (2) and (3) [5, 6]:

$$CE = S_0 \cdot K_{1:1} = \frac{[D/CD]}{[CD]} = \frac{\text{slope}}{(1 - \text{slope})} \quad (2)$$

$$D : CD = 1 : \left(1 + \frac{1}{CE}\right) \quad (3)$$

where $[D/CD]$ is the concentration of the dissolved complex, $[CD]$ the concentration of dissolved free cyclodextrin and the slope is obtained from the phase solubility profile.

2.4 Complex Study by Nuclear Magnetic Resonance

One-dimensional ^1H NMR spectra were recorded on a Varian Inova 500 MHz spectrometer, in unbuffered deuterated water (D_2O) for NFOH, DM- β -CD, and the complex. The stock solutions of NFOH (0.2 mM) and DM- β -CD (0.2 mM) were mixed in 5 mm NMR tubes, giving a total sample volume of 600 μL , and left 32 h for equilibration (stored at 25°C) before the NMR analysis. The probe temperature was regulated to 25°C.

The ^1H NMR spectra were acquired with a spectral width of 6,000 Hz and 32K data-points, (acquisition time 2.730 s), a 90° pulse of 10 μs , and 128 transients. The resonance at 4.81 ppm, due to residual solvent water (H_2O and HDO), was used as internal reference. The data were collected without an external reference to avoid possible interactions with the DM- β -CD. The NMR data were processed with the VNMR 6.1 program (Varian Inc.) using a digital zero filling to 128 K, and a 0.5 Hz exponential function was applied to FID before Fourier transformation.

The relaxation time measurement (T_1) experiments were recorded at 25°C on a Varian Inova 500 MHz NMR spectrometer. The samples were degassed to avoid the interference of dissolved O_2 ; nitrogen was slowly bubbled, for 5 min, through the solution using a thin hard plastic tube (PTFE 1/32-in. diameter). For ^1H NMR, a 90° pulse was typically of 10–15 μs , and the recycling time was set to three times the largest value of T_1 , i.e., 15 s [7]. Longitudinal relaxation times were obtained by the conventional inversion-recovery method.

The DOSY experiments were carried out using the bipolar pulse pair stimulated echo (BPPSTE) method [8–10]. Data was acquired with a 50-ms diffusion delay in all experiments, with a bipolar gradient pulse duration of 2 ms and a 5-ms eddy current delay. The amplitudes of the gradient pulses ranged from 0.000685 to 0.0032 T/cm, where an approximately 90–95% decrease in the resonance intensity was achieved. For all

experiments, 25 different gradient amplitudes were used. The baselines of all arrayed spectra were corrected before data processing. The processing program (DOSY macro in a Varian instrument) involves the determination of the peak heights of all signals above a preestablished threshold and the fitting of the decay curve for each peak. The DOSY macro was run with data transformed using $fn=64$ K. Crowded spectra were processed in sections due to the handling limitation of only 512 lines at a time. The results of the DOSY method of analysis are pseudo two-dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients ($10^{-10} \text{ m}^2 \text{ s}^{-1}$) along the other. The diffusion coefficients were measured for NFOH, DM- β -CD, and NFOH:DM- β -CD.

The NFOH bound fraction was measured as previously described [8, 11]. Assuming that bound and free NFOH undergo fast exchange in the diffusion time scale, the observed NFOH diffusion coefficient, $D_{\text{free+bound}}$, is the weighted average of the unbound, D_{free} , and DM- β -CD-bound, D_{bound} , values (4):

$$D_{\text{free+bound}} = (1 - f_{\text{bound}}) \cdot D_{\text{free}} + f_{\text{bound}} \cdot D_{\text{bound}} \quad (4)$$

where f_{free} and f_{bound} are the fractions of free anesthetic and NFOH bound to DM- β -CD, respectively, and $f_{\text{free}} + f_{\text{bound}} = 1$. D_{free} is the diffusion coefficient of the unbound NFOH, while D_{bound} is that of the DM- β -CD/NFOH complex. Rearrangement of (4) gives:

$$f_{\text{bound}} = \frac{(D_{\text{free}} - D_{\text{free+bound}})}{(D_{\text{free}} - D_{\text{bound}})} \quad (5)$$

Recognizing that NFOH molecular size is much smaller than the one of DM- β -CD, we assumed, as already suggested by other authors [8], that the diffusion coefficient of NFOH bound to the cyclodextrin corresponds to the one of the DM- β -CD alone.

The association binding constant (K_a) of NFOH and DM- β -CD was estimated using the complexed population values previously determined as

$$K_a = \frac{p}{\{(1 - p) \cdot ([CD]_0 - p[S]_0)\}} \quad (6)$$

where p is f_{bound} of (3), and $[S]_0$ and $[CD]_0$ denote the total concentrations of substrate (guest) and cyclodextrin (host), respectively. To account for changes in the solvent viscosity, we found it useful to use D_{2O} diffusion coefficient as an internal reference.

2.5 Release Kinetics Assays

Drug release experiments were conducted under constant stirring, at 37°C, in a two-compartment dialysis system that uses a cellulose membrane (Spectrapore, MWCO 1,000 Da) to separate the sample at the donor compartment (1 mL capacity, containing NFOH (0.2 mM) or NFOH:DM- β -CD (0.2 mM) sample) from the acceptor compartment (100 mL). Aliquots were withdrawn from the acceptor compartment at regular intervals, and NFOH concentration was determined by UV absorption, at 260 nm.

Mathematical modeling (Sigma Plot® 8.0) was used to analyze the release profiles. The profiles were fit with a mono-exponential equation of the form

$$C = C_0 + \alpha \cdot (1 - e^{-k \cdot t}) \quad (7)$$

with observed rate constant k and initial probe concentration C_0 .

3 Results and Discussion

3.1 Kinetics of complexation and phase solubility studies

The complexation phenomenon can be described by parameters such as time to reach solubilization and association constant (K_a) determination [5, 6]. CDs interact with poorly water-soluble compounds to increase their apparent solubility. The most prominent mechanism by which this solubilization occurs is the inclusion complex formation in which the guest and host molecules are in dynamic equilibrium with the complex [12]. The kinetics of complexation between NFOH and DM- β -CD was determined, and it was observed that the complexation equilibrium in aqueous solution was reached up to 20 h (data not shown).

The increased aqueous solubility of NFOH in the presence of DM- β -CD was used to follow the formation of inclusion complexes. The K_a determination between the drug and CD is based on the measurement of an index of changes in physical–chemical properties of a compound upon inclusion. Most methods for determining the K_a values are based on the analysis of the concentration dependencies, at titration experiments with the drug molecule and CD [12]. Thus, an important pharmaceutical application of CD is to enhance drug solubility in aqueous solution. The increase of NFOH solubility occurred as a linear function of DM- β -CD concentration (Fig. 2), corresponding to the AL-type profile defined by Higuchi and Connors [4]. This relationship suggests a first-order kinetics for the complex formation between NFOH and DM- β -CD. The K_a value determined (1) was $58.6 \pm 1.6 \text{ M}^{-1}$, indicating the formation of a stable complex [13]. The increased aqueous solubility of NFOH in the presence of DM- β -CD suggests that these molecules are able to form inclusion complexes.

From (2) and (3) (Section 2), a CE value of 0.0849 was found, which is in agreement with the hydrophobicity of NFOH [6]. Besides, a drug:cyclodextrin ratio of 1:11 was calculated indicating that on a 1:1 (NFOH:DM- β -CD) complexation, just 1 out of 11 DM- β -CD molecules is forming an inclusion complex with NFOH [6].

3.2 Complex Study by Nuclear Magnetic Resonance

The longitudinal relaxation times (T_1) give information about the nucleus mobility in solution. To study the interaction between host and guest molecules, the measurement of T_1

Fig. 2 Phase solubility diagram for NFOH in presence of increasing DM- β -CD concentrations, determined at 25°C. [NFOH]=5 mM. Absorbance is normalized. Data are expressed as mean $\pm$ SD ($n=3$)

can give information about which region of the molecule presents mobility changes after the interaction with another molecule [7]. Table 1 showed the ΔT_1 variation of NFOH hydrogen NMR signals in the presence and absence of DM- β -CD.

Spin-lattice relaxation times (T_1) were measured for NFOH hydrogens before and after the addition of DM- β -CD. A reduction in T_1 values of the nitroheterocyclic group hydrogens of the NFOH molecule is indicative that the movement of the whole molecule is changed in the presence of DM- β -CD [14]. Owens et al. [14] have showed that the reduction in T_1 values for the molecule hydrogens on addition of CD was reported to be due to increased correlation time, τ_c , confirming the restricted rotation of hydrogen atoms as a result of a probable existence of a complex in an aqueous solution. The effect on the T_1 of NFOH hydrogens in the presence of cyclodextrin is caused by the possible interaction with cyclodextrin, as changes in the viscosity of the solution do not occur, once the CD concentration is very small (0.2 mM). Diffusion-ordered spectroscopy (DOSY) NMR experiments were pivotal to demonstrate that NFOH and DM- β -CD form a stable complex. They also serve to distinguish compounds or complexes by their differences in diffusion coefficients [15]. The diffusion coefficient of pure NFOH, NFOH in presence of DM- β -CD and DM- β -CD were $D_{\text{NFOH}}=D_{\text{free}}=4.51 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, $D_{\text{DM-}\beta\text{-CD}}=D_{\text{bound}}=1.37 \pm 0.02 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and $D_{\text{NFOH:DM-}\beta\text{-CD}}=D_{\text{free+bound}}=4.47 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, respectively. In the presence of DM- β -CD, NFOH showed a significant reduction of the diffusion rate, indicating that NFOH formed a *host:guest* complex with DM- β -CD. From these diffusion coefficients and applying a well-established methodology [16], we have calculated the complexed population [percent f_{bound} , (5)] and apparent binding constant [K_a , (6)] of the complex. The value of K_a determined from the interaction between NFOH and DM- β -CD is $65.6 \pm 2.3 \text{ M}^{-1}$. This value was in agreement with the value determined through the solubility isotherm method ($58.6 \pm 1.6 \text{ M}^{-1}$). In all experiments, the water diffusion coefficient was $18.02 \pm 0.01 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, indicating that the presence of NFOH and DM- β -CD (in the concentration used in these experiments) does not change the viscosity of the water. Literature describes that in such low concentration conditions, the viscosity correction is weak, and the difference between apparent and correct diffusion constants is small [17].

3.3 Release Kinetics

Figure 3 showed that complexation of NFOH with DM- β -CD significantly reduced its rate of release from the donor compartment, compared to NFOH in the absence of cyclodextrin. Total release (100%) of NFOH was observed at 200 min of dialysis against the 60% release of NFOH in the presence of DM- β -CD. Gradual drug-release indicates that the addition of carriers, such as CD, changes the drug permeation across the membranes or changes its

Table 1 Values of longitudinal relaxation times (T_1 , s) of NFOH hydrogens in presence of DM- β -CD, [NFOH] = 0.2 mM, at 25°C

NFOH	T_1 without DM- β -CD	T_1 with DM- β -CD
a	4.90 ± 0.2	4.32 ± 0.10
b	4.92 ± 0.56	4.42 ± 0.13
c	3.21 ± 0.12	3.05 ± 0.09
d	—	—

— Not determined

Fig. 3 *In vitro* release profile of NFOH and NFOH:DM- β -CD (1:1 molar ratio) inclusion complex as function of time, determined at 25°C. [NFOH]=0.2 mM. Data are expressed as mean $\pm$ SD ($n=3$)

distribution, with possible therapeutic advantages: prolonged effects, low plasmatic levels, and reduced systemic toxicity [18–22].

The release profile of NFOH in the absence and in the presence of DM- β -CD was modeled using a mono-exponential equation (2). The experimental data adjustment for a mono-exponential fit to NFOH in the presence and absence of cyclodextrin gives a rate constant of $1.75 \pm 0.12 \times 10^{-2} \text{ min}^{-1}$ ($r=0.9998$) and $2.26 \pm 0.20 \times 10^{-2} \text{ min}^{-1}$ ($r=0.9997$), respectively. These results suggest that a different release behavior for NFOH in the absence and presence of cyclodextrin is probably due to complex formation. Related results were published in literature [21–24].

4 Conclusions

The combined use of spectrophotometry and NMR techniques has helped in the determination of the *host:guest* interaction and in the definition of the degree of guest encapsulation in solution, for the NFOH and DM- β -CD inclusion complex. ROESY experiments and molecular modeling are in progress to investigate the *host:guest* structure. The complexation of NFOH with DM- β -CD has been investigated in view of its potential use for the preparation of new therapeutic formulations to increase the bioavailability and decrease the systemic toxicity of NFOH. This study provides perspectives for future experiments using this inclusion complex of NFOH with DM- β -CD aiming to verify its therapeutic efficacy.

Acknowledgments This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (05/03045-9). R.G. and C.M.M. are the recipients of fellowships from FAPESP (06/00787-7 and 04/02091-4, respectively). The authors would like to thank the Synchrotron Light National Laboratory for use of its NMR facility.

References

1. Chung, M.C., Guido, R.V.C., Martinelli, T.F., Gonçalves, M.F., Polli, M.C., Botelho, K.C.A., Varanda, E.A., Colli, W., Miranda, M.T.M., Ferreira, E.I.: Synthesis and *in vitro* evaluation of potential antichagasic hydroxymethylnitrofurazone (NFOH-121): a new nitrofurazone prodrug. *Bioorganic Med. Chem.* **11**, 4779–4783 (2003)

2. Rajewski, R.A., Stella, V.J.: Pharmaceutical applications of cyclodextrins. 2. In vivo drug delivery. *J. Pharm. Sci.* **85**, 1142–1169 (1996)
3. Connors, K.A.: The stability of cyclodextrin complexes in solution. *Chem. Rev.* **97**, 1325–1357 (1997)
4. Higuchi, T., Connors, K.A.: Phase-solubility techniques. *Adv. Anal. Chem. Inst.* **4**, 117–212 (1965)
5. Loftsson, T., Hreindóttir, D., Másson, M.: Evaluation of cyclodextrins solubilization of drugs. *Int. J. Pharm.* **302**, 18–28 (2005)
6. Loftsson, T., Hreindóttir, D., Másson, M.: The complexation efficiency. *J. Incl. Phenom. Macrocycl. Chem.* **57**, 545–552 (2007)
7. Fraceto, L.F., Spisni, A., Schreier, S., de Paula, E.: Differential effects of uncharged aminoamide local anesthetics on phospholipid bilayers, as monitored by H-NMR measurements. *Biophys. Chemist.* **115**, 11–18 (2005)
8. Waldeck, A.R., Kuchel, P.W., Lennon, A.J., Chapman, B.E.: NMR diffusion measurements to characterize membrane transport and solute binding. *Progr. Nucl. Magn. Reson. Spectrosc.* **30**, 39–68 (1997)
9. Fraceto, L.F., Moraes, C.M., Abrami, P., Gonçalves, M.M., Andreo Filho, N., Fernandes, S.A., de Paula, E. Preparação e caracterização Físico-química de complexos de inclusão entre anestésicos locais e hidróxiopropil-beta- ciclodextrina. *Quim Nova* **30**, 777–784 (2007)
10. Jesus, M.B., Pinto, L.M.A., Fraceto, L.F., Takahata, Y., Lino, A.C.S., Jaime, C., de Paula, E. Theoretical and experimental study of a praziquantel and beta-cyclodextrin inclusion complex using molecular mechanic calculations and ¹H-Nuclear Magnetic Resonance. *J. Pharm. Biomed. Anal.* **41**, 1428–1432 (2006)
11. Wimmer, R., Aachamann, F.L., Larsen, K.L., Petersen, S.B.: NMR diffusion as a novel tool for measuring the association constant between cyclodextrin and guest molecules. *Carbohydrate Res.* **337**, 841–849 (2002)
12. Loftsson, T., Brewster, M.E.: Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J. Pharm. Sci.* **85**, 1017–1025 (1996)
13. Loukas, Y.L., Vraha, V., Gregoriadis, G.: Novel non-acidic formulations of haloperidol complexed with beta-cyclodextrin derivatives. *J. Pharm. Biom. Anal.* **16**, 263–268 (1998)
14. Owens, P.K., Fell, A.F., Coleman, M.W., Kinns, M., Berridge, J.C.: Use of ¹H-NMR spectroscopy to determine the enantioselective mechanism of neutral and anionic cyclodextrins in capillary electrophoresis. *J. Pharm. Biomed. Anal.* **15**, 1603–1619 (1997)
15. Johnson Jr., C.S.: Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications progress in nuclear magnetic resonance. *Spectroscopy* **34**, 203–256 (1999)
16. Rymdém, R., Carlfors, J., Stilbs, P.J.: Substrate binding to cyclodextrins in aqueous solution: a multicomponent self diffusion study. *J. Incl. Phenom. Macro.* **1**, 159–168 (1983)
17. Bakkour, Y., Vermeersch, G., Morcellet, M., Boschin, F., Martel, B., Azaroual, N.: Formation of cyclodextrin inclusion complexes with doxycycline-hyclate: NMR investigation of their characterization and stability. *J. Incl. Phenom. Macrocycl. Chem.* **54**, 109–114 (2006)
18. Araújo, D.R., Pinto, L.M.A., Braga, A.F.A., de Paula, E.: Local anesthetic formulation for controlled release: therapeutics applications. *Rev. Bras. Anesthesiol* **53**, 663–671 (2003)
19. Araújo, D.R., Cereda, C.M.S., Brunetto, G.B., Pinto, L.M.A., Santana, M.H.A., de Paula, E.: Encapsulation of mepivacaine prolongs the analgesia provided by sciatic nerve blockade in mice. *Can. J. Anesth.* **51**, 566–572 (2004)
20. Araújo, D.R., Fraceto, L.F., Braga, A.F.A., 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. Anesthesiol.* **55**, 316–328 (2005)
21. Moraes, C.M., Abrami, P., de Paula, E., Braga, A.F., Fraceto, L.F.: Study of the interaction between S(-) bupivacaine and 2-hydroxypropyl-beta-cyclodextrin. *Int. J. Pharm.* **331**, 99–106 (2007)
22. Moraes, C.M., Abrami, P., Gonçalves, M.M., Andreo-Filho, N., Fernandes, S.A., de Paula, E., Fraceto, L.F.: Preparação e caracterização físico-química de complexos de inclusão entre anestésicos locais e hidróxiopropil- β -ciclodextrina. *Quím Nova* **30**, 777–84 (2007)
23. Moraes, C.M., Abrami, P., de Araújo, D.R., Braga, A.F.A., Issa, M.G., Ferraz, H.G., de Paula, E., Fraceto, L.F.: Characterization of lidocaine: hydroxypropyl- β -cyclodextrin. *J. Incl. Phenom. Macrocycl. Chem.* **57**, 313–316 (2007)
24. Araújo, D.R., Tsuneda, S.S., Cereda, M.S., Carvalho, F.D.G.E., Preté, P.S.C., Fernandes, S.A., Yokaichiya, F., Franco, M.K.K.D., Mazzaro, I., Fraceto, L.F., Braga, A.F.A., de Paula, E.: Development and pharmacological evaluation of ropivacaine-2-hydroxypropyl- β -cyclodextrin inclusion complex. *Eur. J. Pharm. Sci.* **33**, 60–71 (2008)