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Clonidine complexation with hydroxypropyl-beta-cyclodextrin: From physico-chemical characterization to *in vivo* adjuvant effect in local anesthesia

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ABSTRACT

Clonidine (CND), an alpha-2-adrenergic agonist, is used as an adjuvant with local anesthetics. In this work, we describe the preparation and characterization of an inclusion complex of clonidine in hydroxypropyl-beta-cyclodextrin (HP-β-CD), as revealed by experimental (UV–vis absorption, SEM, X-ray diffraction, DOSY- and ROESY-NMR) and theoretical (molecular dynamics) approaches. CND was found to bind to HP-β-CD (K_a = 20 M^{-1}) in 1:1 stoichiometry. X-ray diffractograms and SEM images provided evidence of inclusion complex formation, which was associated with changes in the diffraction patterns of the pure compounds. NMR experiments revealed changes in the chemical shift of $H3_{HP\text{-}\beta\text{-}CD}$ hydrogens (Δ = 0.026 ppm) that were compatible with the insertion of CND in the hydrophobic cavity of the cyclodextrin. Molecular dynamics simulation with the three CND species that exist at pH 7.4 revealed the formation of intermolecular hydrogen bonds, especially for the neutral imino form of CND, which favored its insertion in the HP-β-CD cavity. *In vitro* assays revealed that complexation retarded drug diffusion without changing the intrinsic toxicity of clonidine, while *in vivo* tests in rats showed enhanced sensory blockade after the administration of 0.15% CND, with the effect decreasing in the order: CND:HP-β-CD + bupivacaine > CND + bupivacaine > bupivacaine > CND:HP-β-CD > clonidine. The findings demonstrated the suitability of the complex for use as a drug delivery system for clinical use in antinociceptive procedures, in association with local anesthetics.

1. Introduction

Clonidine (2-[2,6-dichloroaniline]-2-imidazoline, CND) was first synthesized in the 1960s. It was originally used as nasal decongestant. However, due to its capacity to lower blood pressure, CND has been successfully employed in the treatment of hypertension for over 25 years. Nevertheless, other uses in clinical practice were proposed [1], and CND has attracted new interest in anesthesiology, as an adjuvant for general and regional anesthesia during surgery or in the postoperative period [2–5]. It is well known that CND prolongs the effect of local anesthetics, reducing the dosage required for anesthesia, with minor adverse effects [6–10]. This beneficial action is due to different mechanisms. In addition to its α_2-agonist action (norepinephrine-like in descending inhibitory pathways), CND acts analogously to a local anesthetic, inhibiting and slowing impulse conduction in C fibers [11,12]. CND also elicits vasoconstriction, mediated by the postsynaptic α_2-receptors, which reduces absorption of the local anesthetic and prolongs its residence time at the neural tissue [6,7]. In summary, the association of clonidine with local anesthetics significantly reduces the time of onset of anesthesia, prolongs the duration and intensity of the sensory block, and leads to sedation due to systemic absorption [13].

Remko et al. [14], using quantum chemical calculations, found that at the CND equilibrium geometry, the imidazole and phenyl rings of the molecule are almost perpendicular to each other, and

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it was suggested that this non-coplanarity is required for the interaction of CND with adrenergic receptors. In addition, it was shown that the imino tautomer, with an exocyclic double bond, is the most stable isomer. Moreover, the primary sites of protonation were found to be in the imidazole nitrogens ($pK_a = 8.0$), with protonated and neutral (amino and imino) forms being present at physiologic pH [15].

Drug delivery systems can be used to manipulate the properties of drugs and enhance their therapeutic effects. Cyclodextrins are among the most promising carriers for the sustained release of antinociceptive agents [16,17], and hydroxypropyl-beta-cyclodextrin (HP-β-CD) has been approved for parenteral use [18,19]. It is well tolerated in humans and, after intravenous administration, is almost completely eliminated via glomerular filtration [20]. HP-β-CD has been extensively studied as a carrier system for local anesthetics, with advantages such as improved solubility and clinical potency [20–25].

This work proposes the complexation of CND with hydroxypropyl-β-cyclodextrin, in order to improve its clinical efficacy in anesthesia. A broad study was undertaken, with preparation and characterization of the complex, followed by *in vitro* and *in vivo* tests, and molecular dynamics simulation, with highly encouraging results.

2. Experimental

2.1. Reagents and chemicals

Clonidine hydrochloride was donated by Cristália Ind. Farm. Ltda. (Itapira, SP, Brazil). Hydroxypropyl-β-cyclodextrin (Kleptose HP®) was purchased from Roquette Serv. Tech. Lab. (Lestrem, Cedex, France). D_2O was obtained from Sigma. DMEM (Dubelcco's Modified Eagle Medium) was acquired from Nutricell (Campinas, SP, Brazil). Bovine fetal serum, penicillin, and streptomycin were obtained from Cultilab (Campinas, SP, Brazil). All other chemicals used were of analytical grade.

3. Methods

3.1. Preparation of the clonidine:HP-β-CD complex

Preparation of the inclusion complex was performed by mixing equimolar amounts of CND and HP-β-CD in water, followed by 12 h agitation, to achieve complete solubilization. The samples were freeze-dried and stored at −20 °C for further use.

3.2. UV-Vis absorption study and stoichiometry determination

The interaction between CND and HP-β-CD was followed by UV absorption in the range 250–310 nm. A titration approach was used to determine the complexation stoichiometry [26], with CND spectra recorded in the presence of increasing HP-β-CD concentrations (CND:HP-β-CD molar ratios of 1:0, 1:1, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, and 1:100). Using the Job plot approach [27], CND spectra were recorded for different CND:HP-β-CD ratios, maintaining a final concentration of CND + HP-β-CD = 2 mM. All experiments were performed in 5 mM Hepes buffer, at pH 7.4 and 25 °C.

The Benesi–Hildebrand procedure was employed to distinguish between the 1:1 and 1:2 stoichiometries, according to [28]:

$$\frac{[\mathrm{CND}]}{\mathrm{Abs} - \mathrm{Abs}_0} = \frac{1}{[\mathrm{HP-\beta-CD}]^n} \quad (1)$$

where [CND] and [HP-β-CD] are the concentrations of CND and HP-β-CD, respectively. Abs (Abs_0) represents the CND absorbance at 271 nm, in the presence (absence) of HP-β-CD, and "*n*" is the stoichiometry of complexation. For the 1:1 stoichiometry, the CND:HP-β-CD association constant (K_a) was calculated from the slope/intercept ratio [28].

3.3. Scanning electron microscopy

Lyophilized samples of the CND:HP-β-CD complex, HP-β-CD, CND, and their physical mixture were deposited on carbon ribbons, previously fixed to aluminum stubs. The stubs were placed in a carbon evaporator (Med 020Coating System, Bal-Tec) and submitted to 3 cycles (20 s) of carbon emission at 75 A. The materials were analyzed with a JSM 500 LV scanning electron microscope, using secondary electron emission.

3.4. X-ray diffraction experiments

Powder diffractograms for samples of HP-β-CD, clonidine, the physical mixture, and the inclusion complex were obtained using a Rigaku wide angle goniometer, equipped with a Cu K_α radiation source (Philips PW 1743), operated at 40 kV and 20 mA. The crystal analyzer was pyrolytic graphite. A scan rate of 1°/min was used, between $2\theta = 5°$ and 60° in a θ–2θ configuration.

3.5. Nuclear magnetic resonance

NMR analyses were performed with a Varian Inova 500 MHz (11.75 T) instrument, at the Brazilian Synchrotron Light Laboratory (LNBio, Campinas, Brazil). One- and two-dimensional ^{1}H NMR spectra were acquired at 25 °C, using D_2O as solvent. Samples (10 mM) of CND, HP-β-CD and the complex were prepared in D_2O at pH 7.4 (adjusted with NaOD and DCl solutions), homogenized for 6 h, and transferred to 5 mm tubes for spectrum acquisition. To avoid any possible interaction with HP-β-CD, no external standards were used [29]; instead, the residual water peak (4.68 ppm) was used as an internal reference.

2D-ROESY experiments were carried out to determine nuclear Overhauser effects (NOE), indicating the spatial proximity between CND and HP-β-CD hydrogens. Rotating-frame cross-relaxations were carried out using spin-locked conditions and NOE in the transverse positive plane. Pulse sequence was employed, with a mixing time of 300 ms [30].

DOSY-NMR spectra were recorded at 25 °C, using the DgcteSL (gradient compensated stimulated echo spin lock) HR-DOSY sequence, as described previously [31]. The amplitudes of the pulsed gradient range were 0.12–0.63 T m^{-1}, where an approximately 90–95% decrease in the resonance intensity was achieved at the largest gradient. For all experiments, 25 different gradient amplitudes were used, with an optimized diffusion time of 0.06 s. The processing program (DOSY macro) was run with data transformed using fn = 32 K.

From the measured diffusion constants (D), the fraction of CND bound to HP-β-CD (f) was determined, according to Eq. (2):

$$f = \frac{D_{\mathrm{CND}} - D_{\mathrm{CND:HP-\beta-CD}}}{D_{\mathrm{CND}} - D_{\mathrm{HP-\beta-CD}}} \quad (2)$$

allowing for the determination of the association constant (K_a), as follows [31]:

$$K_a = \frac{f}{(1-f)([\mathrm{HP-\beta-CD}] - f[\mathrm{CND}])} \quad (3)$$

3.6. Molecular dynamics simulation

Molecular dynamics simulations of the different systems were performed in order to shed light on the interaction between CND and HP-β-CD at the atomic level. Since CND has a pK_a of 8.0 [32],