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Research paper

Characterization of an inclusion complex of cholesterol and hydroxypropyl-β-cyclodextrin

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Abstract

Interactions between endogenous cholesterol and cyclodextrins have been investigated by several researchers, and they found altered skin penetration of some drugs, membrane disruption, and extraction of cholesterol from the large lipoprotein particles or animal fat. In the present study, an inclusion complex composed of cholesterol and hydroxypropyl-β-cyclodextrin (HPβCD) prepared by lyophilization was investigated and characterized in order to confirm these interactions. Five grams of cholesterol were dispersed in 50 ml of 73.2 mM HPβCD aqueous solution, mixed for 2 days, and the filtrate lyophilized. A phase solubility study was performed by mixing an excess amount of cholesterol with an aqueous solution containing increasing amounts of HPβCD. The amount of cholesterol in solution after mixing for 2 days at 25°C was determined by HPLC. The inclusion complex was characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffractometry, and differential scanning calorimetry (DSC). An A_p-type Higuchi phase solubility diagram, DSC, FTIR, and X-ray diffraction demonstrated the formation of an inclusion complex. DSC thermograms indicated that the endothermic peaks of cholesterol and physical mixture of cholesterol with HPβCD due to the fusion of drug crystals, were absent in DSC thermograms obtained on the freeze dried inclusion complex. FTIR spectra indicated that some of the absorption peaks in the lyophilized inclusion complex were different from that of the physical mixture of cholesterol and HPβCD. X-ray diffraction patterns showed that the pure cholesterol and a physical mixture of cholesterol and HPβCD exhibited crystalline characteristics whereas the lyophilized inclusion complex and HPβCD displayed amorphous characteristics. The results indicated that the formation of a cholesterol/HPβCD inclusion complex is more water soluble than cholesterol alone.

Keywords: Cholesterol; Hydroxypropyl-β-cyclodextrin; Inclusion complex; Phase solubility diagram; Fourier transform infrared spectroscopy; X-ray diffractometry; Differential scanning calorimetry

1. Introduction

Cyclodextrins have been used extensively as pharmaceutical excipients to increase the solubility of poorly water soluble drugs by the formation of an inclusion complex between the host cyclodextrin molecule and the guest drug molecule. In addition, studies have been shown to increase stability and bioavailability of drugs [1,2]. Additionally, they can also be used to convert liquid drugs into microcrystalline powders, prevent drug–drug or drug–additive interactions, reduce gastrointestinal or ocular irritation, and reduce or eliminate unpleasant taste and smell in many pharmaceutical preparations [2]. Jabbal Gill et al. [3,4] and Martini et al. [5] found that the undesirable side-effects of the absorption enhancer on cell membranes could be reduced by combining the enhancer with a cyclodextrin. Formation of the inclusion complex involves molecular encapsulation of the guest molecule by the cyclodextrin molecule, and this results in modification of the physicochemical properties, such as solubility and stability of the

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guest molecules. The chemically modified amorphous hydroxypropyl-β-cyclodextrin (HPβCD) has higher water solubility and greater solubilizing and complexing properties than β-cyclodextrin, and is obtained by partial etherification of the crystalline parent cyclodextrins with a hydroxyalkyl group [2,6].

HPβCD can be used safely as a carrier for parenteral delivery of drugs [7,8]. HPβCD is not absorbed from the gastrointestinal tract [9,10]. It is rapidly and nearly completely cleared from the systemic circulation by the kidneys after intravenous injection [11], and is cleared from the lung by being absorbed into the systemic circulation following administration in an aerosol [12]. Parenteral administration of HPβCD was found to cause pulmonary edema in two out of three dogs [13], whereas in rats and monkeys, subacute and subchronic intravenously administered HPβCD did not show any consistent statistically significant alteration in any of the morphological or clinical pathology parameters. Additionally, Brewster et al. also stated that the occasional changes were limited to one sex and/or differed in direction of change between species [8]. More importantly, Carpenter et al. presented systemic evidence of the absence of any complications for up to 8 years after the intravenous administration of HPβCD to humans [14,15]. Recently, the aqueous inclusion complexes of benzothiophene compounds with HPβCD in intravenous formulation, aerosol solution and oral formulation have been patented for prevention of bone loss and lowering serum cholesterol levels [16].

Cholesterol is a principal sterol synthesized in the liver and generally found in all body tissues [17]. Also it is the precursor of steroid hormones such as progesterone, testosterone, estradiol, and cortisol [18]. Even though cholesterol has been widely used as an emulsifying agent and solubilizing agent in many pharmaceutical applications, such as emulsions and ointment bases [19], its use is limited by its lack of solubility in aqueous media. Interactions between cholesterol and cyclodextrins have been described by several researchers. Irie et al. reported that the inclusion of membrane components such as cholesterol, protein and phospholipid with cyclodextrin, induced hemolysis which resulted in a membrane disruption [20]. Okamoto et al. investigated the effect of β-cyclodextrin and di-*O*-methyl-β-cyclodextrin on skin penetration. They reported a decrease in skin penetration of butylparaben and indomethacin whereas an increase was found for sulfanilic acid with both cyclodextrins. An increase in skin penetration of sulfanilic acid was due to the effect of both cyclodextrins on decreasing the skin barrier function resulting in the significant acceleration of penetration [21]. Frijlink et al. found that complex formation of endogenous cholesterol with β-cyclodextrin and hydroxypropyl-β-cyclodextrin in the bloodstream leads to extraction of cholesterol from the large lipoprotein particles [22]. This interaction may be a concern when a parenteral formulation of a drug- HPβCD complex is administered intravenously since drug bioavailability and therapeutic efficacy may be influenced by the interaction of endogenous cholesterol with HPβCD present in the formulation.

The objective of the present study was to investigate the interactions between cholesterol and HPβCD as confirmed in previous clinical studies. The solubility enhancement of cholesterol by inclusion complexation with HPβCD also was evaluated. Finally, the formation of such a complex was confirmed by phase solubility studies, Fourier transform infrared spectroscopy (FTIR), X-ray diffractometry, and differential scanning calorimetry (DSC).

2. Materials and methods

2.1. Materials

Cholesterol ($C_{27}H_{46}O$; molecular weight, 386.7; melting point, 148.5°C) was obtained from Sigma (Sigma grade: >99% purity, St. Louis, MO, USA). Hydroxypropyl-β-cyclodextrin (HPβCD, Encapsin®) was purchased from American Maize-Products/Cerestar USA (Hammond, IN, USA). Potassium bromide (KBr) was obtained from EM Science (Gibbstown, NJ, USA). Isopropanol (HPLC grade, EM Science) was used to prepare the mobile phase and dilutions for spectrophotometry. All materials were used as received. Purified water (Milli QUV plus water system, Millipore, Molsheim, France) was used.

2.2. Methods

2.2.1. Preparation of the inclusion complex

The inclusion complex between cholesterol and HPβCD was prepared by lyophilization. Five grams of cholesterol were dispersed in 50 ml of 73.2 mM HPβCD aqueous solution and mixed for 2 days at room temperature. The suspension was filtered through a 0.45 μm PTFE filter (Acrodisc® 13 CR PTFE, Gelman Sciences, Ann Arbor, MI, USA). The filtrate was frozen and then lyophilized (Labconco Freeze Dry System/Freezone® 4.5, Labconco, Kansas City, MO, USA).

2.2.2. Preparation of the physical mixture

A physical mixture consisting of cholesterol and HPβCD in the same weight ratio as the lyophilized complex was prepared. The cholesterol and HPβCD were admixed together in a mortar and pestle for 5 min to obtain a homogeneous blend.

2.2.3. Phase solubility studies

Phase solubility studies were carried out according to the method described by Higuchi and Connors [23]. An excess amount of cholesterol was mixed in an aqueous solution containing increasing amounts of HPβCD (0–73.2 mM) using a laboratory shaker (Lab-Line® Orbit Environ-shaker, Lab-line Instruments, Melrose Park, IL, USA) at 25°C. The amount of cholesterol in solution after equilibrating for

2 days was determined by HPLC analysis. The samples were prepared for HPLC analysis by filtering through a 0.45 μm PTFE filter. Appropriate dilutions were made with isopropanol such that the final concentration was within the linear portion of the standard curve for cholesterol prior to injection onto the HPLC column.

A suitable HPLC analytical method was developed for quantitating the amount of cholesterol in solution. The chromatographic system (Shimadzu, Columbia, MD, USA) consisted of a solvent delivery module (Model LC 9A), variable-wavelength UV spectrophotometric detector (Model SPD-6A), chromatographic data control and acquisition system (CLASS-VP), and LC-personal computer (PC) interface (Model LPI 6B). A Zorbax ODS (Zorbax, Du Pont, DE) column (4.6 mm 25 cm) was used.

The mobile phase consisted of purified water and isopropanol (80:20, v/v), and was deaerated prior to use. The flow rate was 2 ml/min and the injection volume was 40 μl. The absorbance was monitored at 200 nm. System suitability was performed for each HPLC analysis, and consisted of specificity, linearity, accuracy, and repeatability. System suitability confirmed that the operating system was performing properly.

2.2.4. Differential scanning calorimetry (DSC)

DSC was performed using a Modulated Differential Scanning Calorimeter (Model DSC 2920, TA Instruments, New Castle, DE, USA). The modulated DSC (reverse heat flow as a function of temperature) was chosen instead of conventional DSC (heat flow as a function of temperature) because it provides unique capabilities including improvement of sensitivity for subtle transitions as well as separation of reversing and non-reversing characteristics of thermal events [24]. The scan rate was 10°C/min in a dynamic nitrogen environment between 80°C and 230°C. The sample weighed 2–4 mg and was contained in closed aluminum pans. Duplicate determinations were carried out for each sample.

2.2.5. Fourier transform infrared spectroscopy (FTIR)

FTIR was conducted using a Nicolet Magna IR Spectrometer (Model 550, Nicolet, Madison, WI, USA). The diffuse reflectance technique was utilized in the mid-IR (400–4000 cm) spectral region. The procedure consisted of placing a sample of the neat powder dispersed in KBr (about 200–400 mg) into the sampling cup, smoothing the powder into a thin bed, and compressing the powder bed into the holder using a compression gauge. The sample was placed in the light path and the spectrum was obtained. FTIR was performed in duplicate for each of the samples.

2.2.6. Powder X-ray diffractometry

The powder samples were packed in the X-ray holder from the top prior to analysis. X-ray diffraction patterns were obtained with a Philips vertical scanning diffractometer (Type PW1729, Philips Electronic Instruments, Mount Vernon, NY, USA) and Philips data acquisition software (Type APD3520) over a 2-theta range of 5° to 50° (where theta is the scattering angle) with a step size of 0.05° 2-theta. The analysis was carried out at room temperature under ambient conditions. Duplicate determinations were made for each of the samples.

3. Results and discussion

A phase solubility study was performed by gently shaking the vials containing an excess amount of cholesterol and solutions of HPβCD in which the concentration ranged from 0 to 73.2 mM. It was observed that at high concentrations of HPβCD, the supernatant was more turbid with less cholesterol settling to the bottom of the vial, possibly due to the enhanced wetting of excess cholesterol particles at the higher concentrations of HPβCD. This was due to an increase in the solubility of cholesterol by forming an inclusion complex with the HPβCD. Fig. 1 shows the phase solubility diagram of cholesterol and HPβCD after analyzing the filtrate of the supernatant of the mixture. It was observed that the solubility of cholesterol increased as the concentration of HPβCD was increased. The equilibrium solubility of cholesterol, S_o, was taken from the literature (0.002 mg/ml; [17]), since the very low concentration of cholesterol was below the detection limits of the HPLC method used in the present study. The phase solubility diagram of cholesterol with HPβCD resulted in an A_p-type Higuchi phase solubility diagram (r^2 from linear regression of 0.991) similar to that reported by Frijlink et al. [22] and Higuchi and Connors [23]. The curve showed a positive deviation from the straight line of the A_L-type where complexes formed were of the first order with regards to the host molecule. Therefore, the complexes formed, in the A_p-type Higuchi phase solubility diagram were presented to a higher order than one in the host molecule (i.e. HPβCD). Additionally, this may indicate the formation of 1:1 and 1:2 stochio-

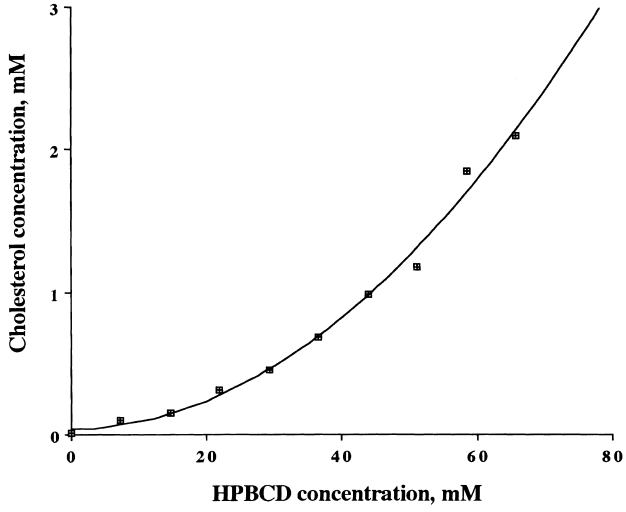

Fig. 1. Higuchi phase solubility diagram of HPβCD and cholesterol.

metric ratios of cholesterol/HPβCD complexes [23]. At the higher concentrations of HPβCD, complexation between more than one HPβCD molecule and one guest molecule (i.e. cholesterol) was likely to have occurred. Due to the very low undetectable cholesterol concentration in aqueous solution in the absence of HPβCD (equilibrium solubility of cholesterol, S_o) by HPLC, the complex formation or stability constant for this system could not be determined from the phase diagram directly. The same limitation was previously described by Frijlink et al. [22], that the low aqueous solubility of the pure cholesterol made the determination more difficult since cholesterol may be disturbed by self-association of cholesterol in the solution [25].

The DSC results presented in Fig. 2a demonstrate an endothermic peak for cholesterol at 152°C, which corresponded to the melting point. The melting point reported for pure cholesterol is 148.5°C (Merck Index, Sigma Library) [17,26] and within the range of 148°C to 150°C (TRC Data Series) [27]. Similar results were seen for the physical mixture of cholesterol and HPβCD in Fig. 2c. The physical mixture thermogram was nearly identical to that of pure cholesterol, and showed a strong endothermic peak at approximately 152°C. The DSC thermogram of HPβCD observed in this study was similar to previously published studies [28,29]. As can be seen in Fig. 2b,d, the thermogram of HPβCD and of the inclusion complex did not show any

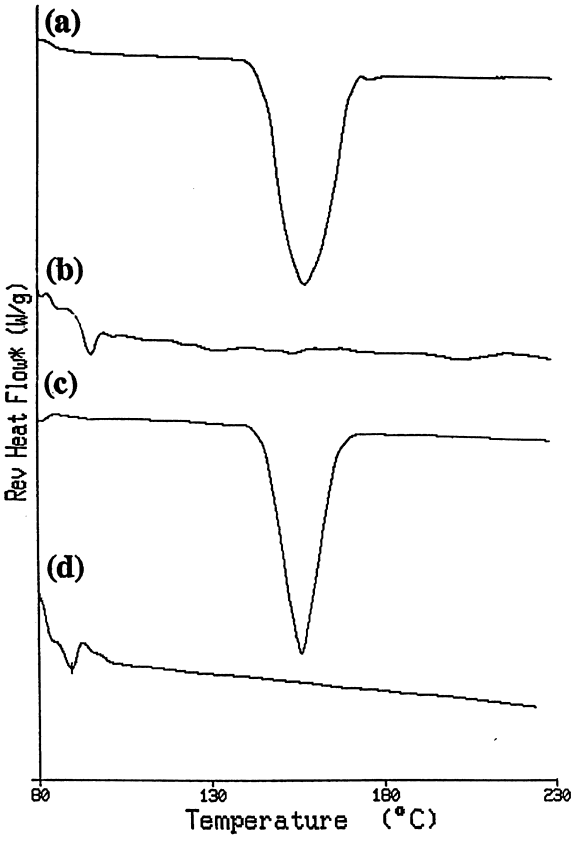

Fig. 2. DSC thermograms of (a) cholesterol, (b) HPβCD, (c) physical mixture, (d) inclusion complex.

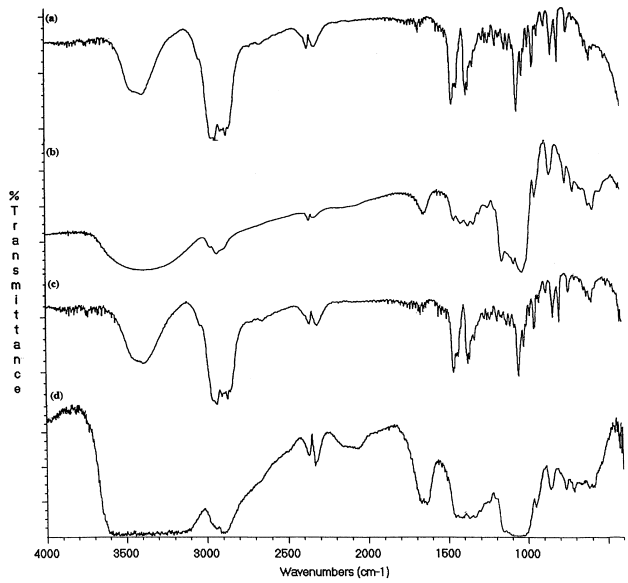

Fig. 3. FTIR Spectra of (a) cholesterol, (b) HPβCD, (c) physical mixture, (d) inclusion complex.

sharp endothermic peak in the temperature range investigated. This indicated the amorphous character of both samples. No cholesterol peak was detected. The disappearance of the endothermic peak from the thermogram obtained for cholesterol compared with the thermogram obtained for the complex indicated that the freeze drying technique produced an inclusion complex between cholesterol and HPβCD, not a simple physical mixture. Therefore, the endothermic peak of cholesterol was not detected since the crystalline cholesterol molecule was contained within the cavity of the HPβCD ring molecule.

FTIR is a useful technique used to confirm the formation of an inclusion complex. Ahmed et al. used FTIR to support the evidence for a complex formation between bropirimine and β-cyclodextrin in solution and in the solid state [30]. The FTIR spectrum of the physical mixture shown in Fig. 3c showed no significant differences from the respective spectra of each of the pure components as seen in Fig. 3a,b. However, some significant differences could be seen in the FTIR spectrum of the inclusion complex as displayed in Fig. 3d. FTIR spectroscopy of the cholesterol-HPβCD inclusion complex indicated very small absorption peaks characteristic of the C-H stretching in the range of 2800 to 3000 cm and C-O bond of saturated secondary alcohol in the range of 1000 to 1100 cm for the pure cholesterol and the physical mixture. Additionally, the broader peak of O-H stretching in the range of 3000 to 3600 cm was found from the inclusion complex spectra, and this corresponded to the multiple O-H functional groups of HPβCD molecules as compared to the narrow peak of O-H stretching from the pure cholesterol spectra. This result was consistent with the results obtained from X-ray diffraction, DSC and the phase solubility study.

Further evidence of complex formation was obtained by X-ray powder diffraction as demonstrated in Fig. 4. The X-

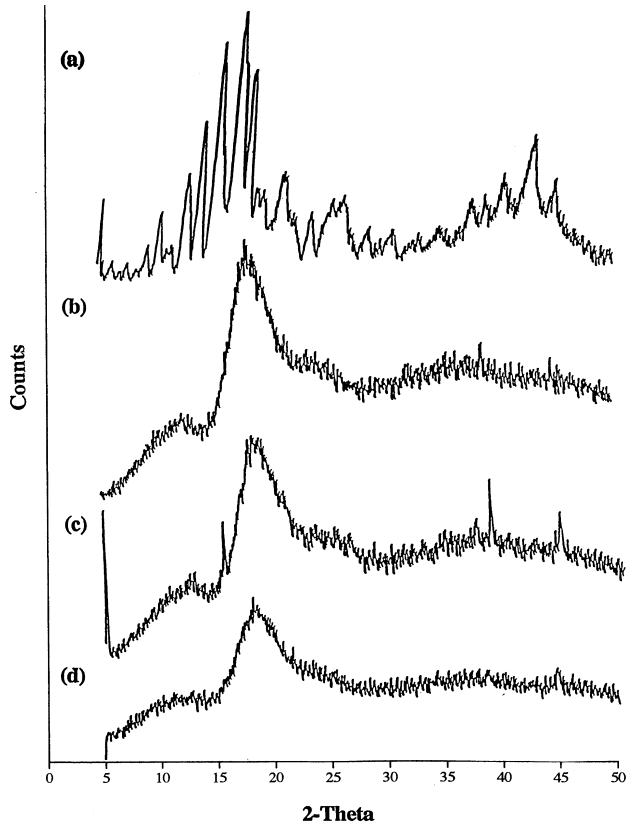

Fig. 4. X-ray diffractograms of (a) cholesterol, (b) HPβCD, (c) physical mixture, (d) inclusion complex.

ray powder diffraction patterns of pure cholesterol shown in Fig. 4a displayed crystallinity whereas an amorphous pattern lacking crystalline peaks was observed for HPβCD as shown in Fig. 4b. The diffractogram of the physical mixture shown in Fig. 4c was the superimposed figures of each of the pure components with the peaks having a lower intensity. The diffractogram consisted mostly of the HPβCD character, but some of the cholesterol characteristics remained. This was due to a reduction in particle size during the preparation of the physical mixture and to the dilution of the cholesterol in the physical mixture. When compared to the diffraction patterns of pure cholesterol and HPβCD, the diffractogram of the inclusion complex shown in Fig. 4d was superimposable with that of the amorphous HPβCD shown in Fig. 4b. This lack of crystallinity provided evidence for the formation of an inclusion complex.

The solid state properties of the inclusion complex that was prepared by lyophilization was stable after storage for 6 months at 4°C. The DSC thermogram indicated that the inclusion complex retained its amorphous characteristic with absence of the endothermic peak after storage. Similarly, the X-ray powder diffraction pattern displayed identical amorphous patterns which lacked the characteristic crystalline peaks of cholesterol.

In conclusion, the interaction between cholesterol and HPβCD resulted in formation of an inclusion complex formation with enhanced aqueous solubility compared to cholesterol. The formation of the inclusion complex was investigated and confirmed by phase solubility studies, DSC, FTIR, and X-ray diffraction.

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