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In vitro and *in vivo* evaluation of an amylobarbitone/hydroxypropyl-β-cyclodextrin complex prepared by a freeze-drying method

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The complex formation of amylobarbitone (AMB) with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was investigated in aqueous solution and in the solid state. The apparent stability constant for complex formation (K_c) calculated by phase solubility and spectral shift methods was 524 M^{-1} and 568 M^{-1}, respectively. The stoichiometric molar ratio of the complex was estimated to be 1 : 1 and the solubility of AMB in water was increased about 3 fold. The solid dispersion system of AMB/HP-β-CD in 1 : 1 molar ratio was prepared by a freeze-drying method. Differential scanning calorimetry (DSC), x-ray diffractometry, (IR) and ^{1}H NMR spectroscopy were used to confirm that inclusion between the drug and HP-β-CD occurred. The dissolution behavior of the drug as a physical mixture as well as the prepared complex, showed enhanced drug dissolution properties of the prepared complex compared to the physical mixture or the drug alone. The dissolution rate appeared in the first 2 min, 25 times greater for the complex than for the drug alone. Furthermore, *in-vivo* study revealed that the duration and hypnotic activity of AMB after its oral administration to mice were improved by inclusion.

1. Introduction

The formation of an inclusion complex of a drug with non-toxic agents is a type of manipulation used to improve the dissolution properties of drugs [1]. Cyclodextrins are cyclic carbohydrates capable of forming this type of complexation by enclosing the drug molecules in their hydrophobic cavity or in a channel formed by several molecules, and they have thus been utilized in pharmaceutical formulations to improve the chemical stability and bioavailability of many pharmaceuticals [2–6]. HP-β-CD is a highly water-soluble amorphous cyclodextrin which has the ability to form inclusion complexes without any significant toxicity or irritability by various administration routes [7–9]. Amylobarbitone, AMB, a short acting hypnotic, is used for treatment of insomnia and for routine sedation and to relieve anxiety. Also, it may be used i.v. or i.m. to control status epilepticus or acute seizure episodes resulting from meningitis, poisons, tetanus or cholera [10]. The limited water solubility has been found to restrict the incorporation of the drug in various pharmaceutical formulations. Reportedly, AMB sodium solutions decompose on standing [11, 12]. In addition, AMB may be precipitated from preparations containing AMB sodium, depending on the concentration and pH. Moreover, the drug is incompatible with many other drugs such as acids and acidic salts. Furthermore, necrosis may result following subcutaneous injection of the highly alkaline AMB sodium solution [12].

The present study of inclusion complexation of AMB with HP-β-CD therefore aimed to enhance solubility, and dissolution properties, and consequently improve its pharmacological activity.

2. Investigations, results and discussion

The phase-solubility diagram of AMB in aqueous HP-β-CD solutions at 30 °C is presented in Fig. 1. This plot shows that the aqueous solubility of the drug increases linearly ($r = 0.998$) as a function of HP-β-CD concentration. It is clear that the solubility diagram of AMB in the presence of HP-β-CD can be classified as A_L-type. The linear relationship may be ascribed to the formation of a soluble 1 : 1 AMB/HP-β-CD complex at the used range of HP-β-CD concentrations (0.001–0.01 M). The apparent stability constant (K_c) was found to be 524 M^{-1} according to Higuchi and Connors' equation [13]. This value of K_c was, in the range of 200 to 5000 M^{-1}, considered by various authors to be adequate for the formation of an inclusion complex, which may contribute to the improved bioavailability of poorly water-soluble drugs [14]. However, the classic method using solubility diagrams requires calculations involving drug solubility, and so could be impaired by low drug solubility values. The stability constant was therefore also assessed by the spectral shift method. The changes in absorption spectra of AMB in the presence of different molar concentrations of HP-β-CD showed a bathochromic shift in the absorption maxima of the drug with a hypochromic effect. These changes in absorbance can be attributed, primarily, to the formation of the inclusion complex in the liquid state. The observed reduction in peak intensity may be the result of the transfer of guest molecules from water to the cavity of the CD molecule. This is reasonable in view of the fact that there

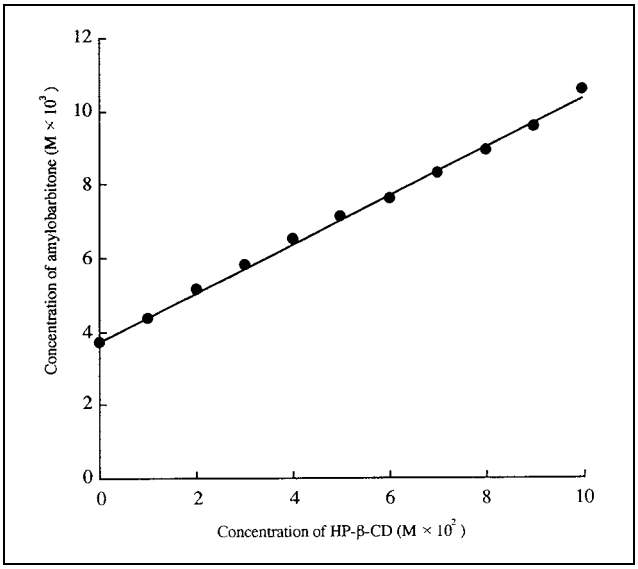


Fig. 1: Phase solubility diagram of amylobarbitone and HP-β-CD in water at 30 °C

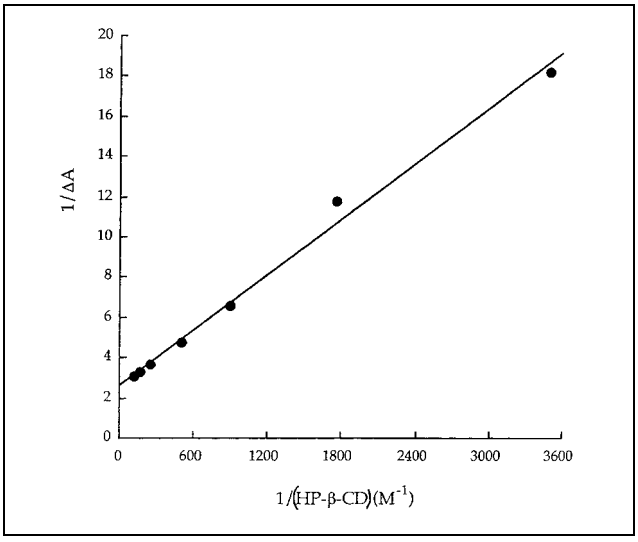


Fig. 2: Benesi-Hildebrand plot for AMB/HP-β-CD system – ΔA is the change of absorbance at $\lambda_{max} = 252$ nm. (HP-β-CD) is cyclodextrin concentration

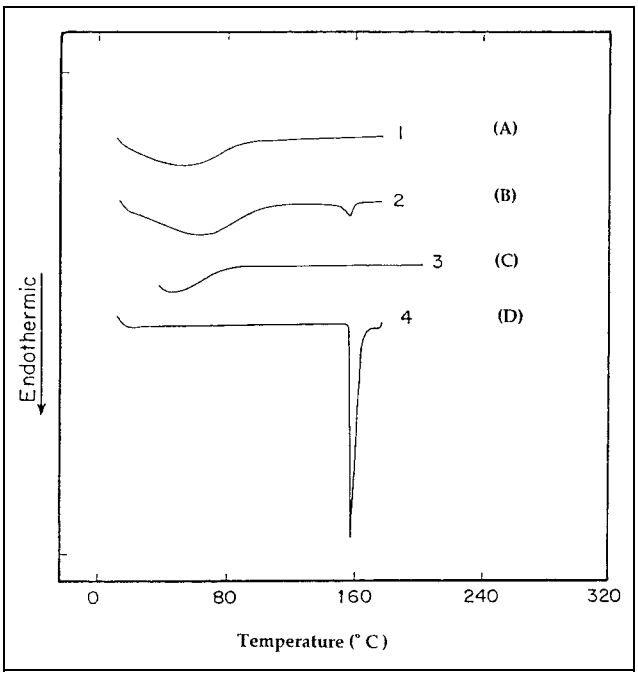


Fig. 3: Differential scanning calorimetry thermograms of:
A: amylobarbitone/HP-β-CD complex B: physical mixture
C: HP-β-CD D: amylobarbitone

are no proton donating groups inside the cavity [15]. When the spectral data were analyzed by the double reciprocal plot as shown in Fig. 2, the plot of 1/ΔA vs 1/[CD] is linear ($r = 0.996$) indicating the presence of a 1 : 1 complex. The stability constant was found to be 568 M^{-1} which is in agreement with the value calculated by the solubility method.

More evidence for complex formation was provided by Differential Scanning Calorimetry (DSC). The DSC thermograms (Fig. 3) showed that the drug melts with an endothermic peak having an enthalpy of fusion (ΔH) of −100.82 J/g, and a thaw point of 152 °C while, the peak melting point was 157.1 °C. In the case of a physical mixture, two peaks appeared, the first peak, at around 85 °C, which could be attributed to the crystalline or adhesional-water molecules in HP-β-CD, and the second one with a ΔH of −8.06 J/g, a thaw point of 142.4 °C and the peak melting point at 155.5 °C suggesting a relative reduction in the crystallinity of AMB. In contrast, this endothermic peak was completely absent in freeze-dried samples suggesting the formation of a new amorphous compound by inclusion of the drug molecules in the cavity of HP-β-CD.

The powder x-ray diffraction patterns of the complex in comparison with those of the physical mixture and the drug alone are displayed in Fig. 4. AMB and HP-β-CD presented well defined crystalline x-ray patterns. Patterns of the physical mixture demonstrated a simple superposition of each component, while that of the freeze-dried sample was clearly different showing that a new solid phase had been formed. The AMB/HP-β-CD complex gave a diffuse diffraction pattern suggesting that it is much less crystalline than the simply mixed counterpart.

IR spectra supported the previous results and confirmed the formation of an AMB/HP-β-CD inclusion complex by the freeze-drying method. AMB (Fig. 5) is characterized by peaks appearing between 1750 and 1400 cm^{-1}, which cannot be confused with HP-β-CD peaks around 1200–1000 cm^{-1}. The bands at 1714 and 1690 cm^{-1}, characteristic of AMB carbonyl stretching bands, were retained in the physical mixture, whereas they had almost disappeared in case of the inclusion complex.

The ^1H NMR technique (400 MHz) was employed to examine the inclusion mode of AMB and HP-β-CD. The chemical shift changes ($\Delta\delta = \delta_{complex} - \delta_{free}$) for the HP-β-CD protons inside the complex, with respect to the HP-β-CD alone, were as follows:

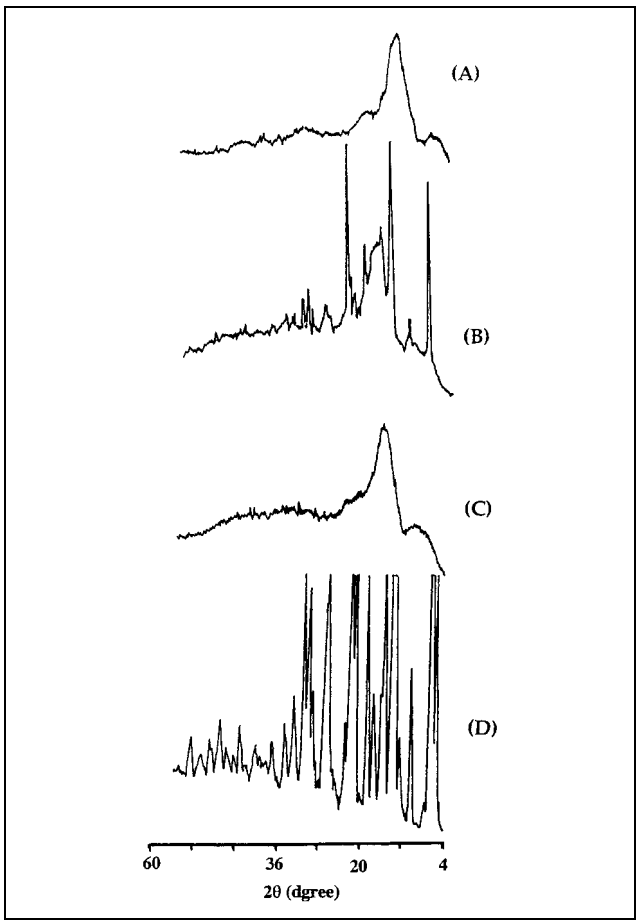
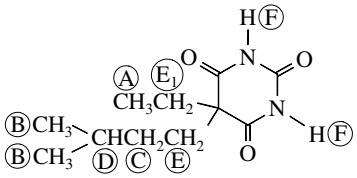


Fig. 4: Powder X-ray diffraction pattern of:
A: amylobarbitone/HP-β-CD complex B: physical mixture
C: HP-β-CD D: amylobarbitone

Table: Effects of HP-β-CD on chemical shifts of amylobarbitone protons



Proton	Multiplicity	Chemical shift (ppm)	
		without HP-β-CD	with HP-β-CD
Ⓐ	Triplet	0.69, 0.71, 0.72	0.69, 0.71, 0.74
Ⓑ	Doublet	0.75, 0.77	0.78, 0.79
Ⓒ	Multiplet	0.896, 0.91, 0.92, 0.94, 0.95	0.90, 0.92, 0.95, 0.96, 1.01 (1.01 due to signal of CD)
Ⓓ	Multiplet	1.33, 1.35, 1.36, 1.38, 1.40, 1.41, 1.43	1.37, 1.38, 1.42, 1.43
Ⓔ+Ⓔ$_1$	Multiplet	1.73, 1.74, 1.75, 1.77, 1.79, 1.80, 1.82	1.74, 1.75, 1.76, 1.77, 1.78, 1.79, 1.81, 1.83
Ⓕ	Singlet	11.43 (sharp signal)	11.48 (broad signal)

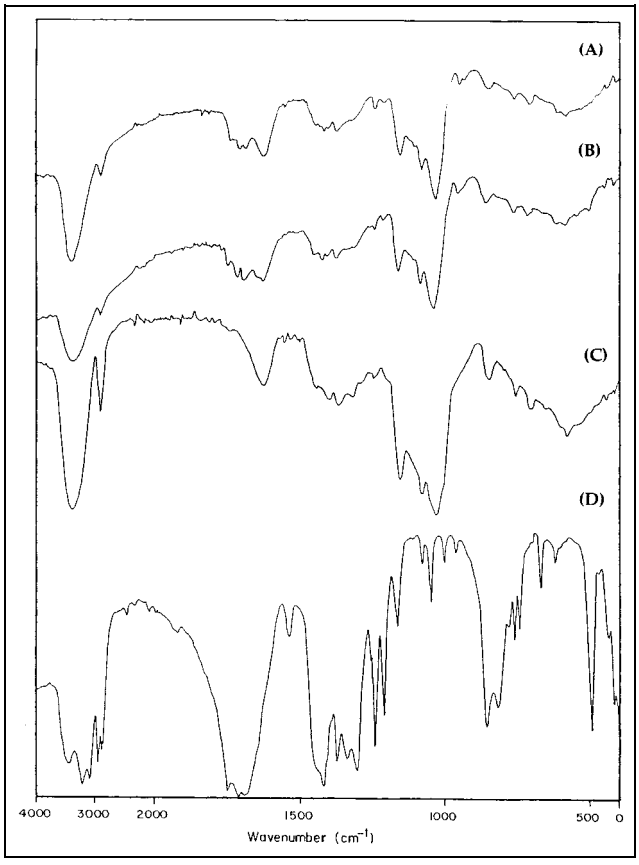


Fig. 5: Infrared spectra for:
A: amylobarbitone/HP-β-CD complex B: physical mixture
C: HP-β-CD D: amylobarbitone

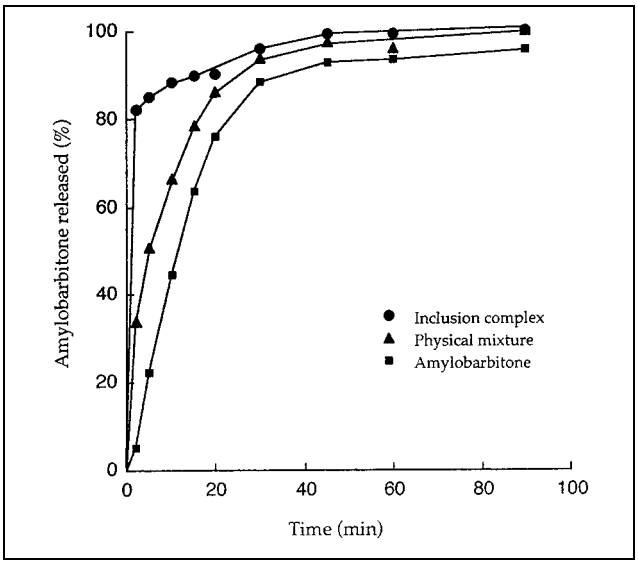


Fig. 6: Dissolution profiles of amylobarbitone from its different systems in water at 37 °C

-0.0049, $+0.0025$, -0.0122, -0.0013, -0.0013, $+0.0024$ for H_1, H_2, H_3, H_4, H_5, H_6 respectively. Therefore, the shifts of the proton signals located within the cavity (specially H_3) of HP-β-CD to higher fields suggested that a hydrophobic interaction was predominant between the drug and HP-β-CD. The Table summarizes the effects of HP-β-CD on the ^{1}H-chemical shifts of AMB. In the presence of HP-β-CD, downfield shifts were observed for all proton signals of AMB probably due to steric perturbation through inclusion complexation [16].

Fig. 6 shows the dissolution profiles of the drug, a physical mixture and the prepared complex in water at 37 °C. It is evident that both the physical mixture and complex demonstrated a significant enhancement in the dissolution rate compared to the drug alone. The amount of AMB (%) dissolved at 2 min was about 78, 37, 5 for the complex, physical mixture and drug alone, respectively. The enhanced dissolution rate can be attributed to an increase in solubility and a decrease in crystallinity. An improvement in the wettability of amylobarbitone particles due to rapid dissolution of HP-β-CD in the microenvironment is believed to account for the increased dissolution rate of the physical mixture [17].

The *in-vivo* performance of AMB was studied by estimating the onset and duration of its hypnotic effect. Fig. 7 shows the onset and duration of hypnosis by AMB after oral administration in the free form and in the inclusion complex with HP-β-CD to five mice. The shorter onset and longer duration of hypnosis with the inclusion complex than with the drug alone suggests that the formation of the inclusion complex of AMB with HP-β-CD improved the pharmacological activity of the drug by increasing its bioavailability. Koizumi et al. [18] reported that the sleeping lag (time from oral adminstration to loss of righting reflex) on administration of the complex to

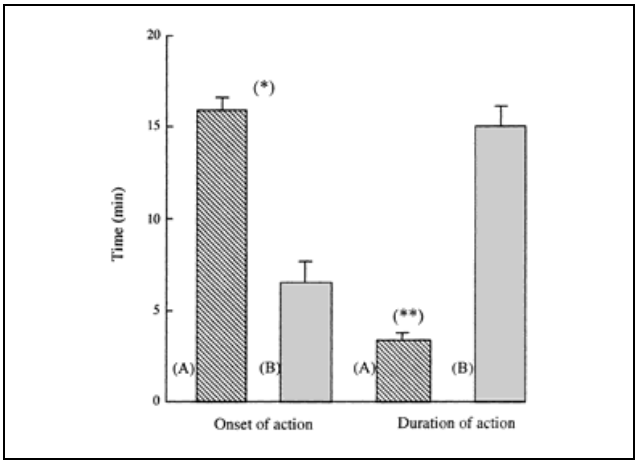


Fig. 7: Mean onset and duration of hypnotic action, ±S.D., of amylobarbitone after oral administration of 1.6 mg/animal of the drug (A) and its inclusion complex (B) to five mice
* $P = 0.0035$
** $P < 0.0001$

mice was shorter than when an equimolar amount of the intact drug was given, and the sleeping time (time from loss to recovery of righting reflex) was significantly increased by inclusion complexation with β-cyclodextrin. The results were attributed to acceleration of the absorption of AMB in the stomach by complexation through enhancement of the solubility and dissolution rate of the drug in acidic pH. At the same time, complexation significantly increased the sleeping time. They assumed that complexation may increase the amount of AMB reaching the brain and also tends to maintain the brain level of the drug for a longer time. However, as noted by Uekama et al. [19], it is also possible that cyclodextrins may affect drug absorption through modification of the mucosal membrane. Free cyclodextrin may remove membrane components [20, 21], thereby modifying the transport properties of the membrane and facilitating absorption of the drug.

In conclusion, amylobarbitone was found to form an inclusion complex with HP-β-CD producing an A_L type phase solubility diagram. The apparent stability constant for complex formation (K_c), calculated by phase solubility and spectral shift methods, respectively, was 524 M^{-1} and 568 M^{-1}. This value of K_c has been considered by various authors to be adequate for the formation of an inclusion complex, which may contribute to the improved aqueous solubility of the poorly soluble drug AMB. This improved aqueous solubility caused an increased *in-vivo* dissolution rate of the drug and hence faster onset and longer duration of hypnotic action than with the drug alone when they were administered orally to mice. In general, these results showed that HP-β-CD is useful additive for increasing the aqueous solubility and bioavailability of AMB.

3. Experimental

3.1. Materials

AMB was obtained from Sigma Chemical Co, USA. HP-β-CD with a molecular weight of 1541 (corresponding to a degree of substitution of seven 2-hydroxypropyl residues per molecules); was provided by American Maize-Products Co., Hammond, IN, and used as recieved. All other chemicals were of reagent grade.

3.2. Methods

3.2.1. Phase-solubility study

The study was performed according to the method of Higuchi and Connors [13]. AMB, in amounts that exceeded its water solubility (100 mg), was accurately weighed into 50 ml Erlenmeyer flasks to which were added 20 ml of water containing various concentrations of HP-β-CD (0.001–0.01 M). The flasks were shaken at 100 strokes/min and 30 °C for 72 h, a time considered enough to reach equilibrium, using a shaker with a thermostatically controlled water bath. The suspensions were then filtered using a millipore filter (0.45 μm). An aliquot portion of each filtrate was spectrophotometrically analyzed for its drug content using a Shimadzu double beam spectrophotometer UV-150-02, Japan, at 252 nm after appropriate dilution with 0.1 N NaOH. The presence of trace amounts of HP-β-CD did not interfere with the assay. The apparent stability constant (K_c) for the complex formed was calculated from the slope of the phase-solubility profile and the aqueous solubility of AMB (S°) according to the following equation:

$$K_c = \text{Slope}/S^\circ \quad (1\text{-Slope})\,.$$

3.2.2. Spectral shift

The spectral shift method was used to study the formation of a complex between AMB and HP-β-CD [22]. The concentration of the drug was 2×10^{-4} M, while concentrations of HP-β-CD ranged from 0 to 8×10^{-3} M. UV spectra of AMB recorded on a Unicam SP 1750 UV spectrophotometer. The change of the absorbance of AMB by the addition of various concentrations of HP-β-CD was measured at 252 nm in 0.1 N NaOH. The apparent stability constant of the complex, was calculated from the spectral data according to the Benesi-Hildebrand equation [23]:

$$\frac{1}{\Delta A} = \frac{1}{[D]\,\Delta\varepsilon} + \frac{1}{[D]\,K_c\,\Delta\varepsilon}\,\frac{1}{[CD]}$$

where ΔA is the difference of absorbance at 252 nm, [CD] is HP-β-CD concentration, [D] is the total drug concentration and Δε is the difference of molar absorptivities between the complexed and free drug. The stability constant (K_c) was obtained from the intercept/slope ratio of the linear plot of 1/ΔA vs 1/[CD].

3.2.3. Preparation of AMB/HP-β-CD complex by a freeze-drying method

Equimolar amounts of AMB and HP-β-CD were dissolved in a suitable amount of distilled water using ultrasonic dispersion until a clear solution was obtained. The aqueous solution was lyophilized in Labconco, Freeze Dry/Shell Freeze System, Freezone-6 (Labconco Corporation, Kansas City, Miswsouri 64132). The product was kept in a desiccator for 48 h. The drug content in the freeze-dried sample was determined spectrophotometrically. A physical mixture of AMB with HP-β-CD, at 1 : 1 molar ratio, was prepared by simple blending.

3.2.4. Instrumental analysis

3.2.4.1. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC-50 Shimadzu, Japan) was performed under the following conditions: sample weight 3–5 mg, scanning rate 10 °C/min, N_2 purge 30 ml/min. The instrument was calibrated for temperature and energy with pure indium (99.999%, m.p. 156.6 °C, transition energy 28.45 J/g). Thermal analysis data were obtained using a TA 50I PC system with Shimadzu software programs. Those programs allow the highest levels of calorimetric accuracy and reproducibility of heat flux DSC (1% and 0.1% respectively).

3.2.4.2. X-ray diffractometry

Powder X-ray diffraction patterns for all samples were obtained using a diffractometer (X-ray diffractometer, PW 1700/1710, Philips, Netherlands) under the following conditions; X-ray, Ni filtered Cu-kα radiation; voltage 40 kV; current 30 mA; scanning speed 10 mm/sec.

3.2.4.3. Infrared absorption spectroscopy (IR)

The IR spectra of AMB, the inclusion complex, a physical mixture and HP-β-CD were carried out using a Hitachi 295 spectrophotometer. The samples were prepared as KBr disks compressed under a pressure of 6 ton/cm^2. The wave number selected ranged between 400 and 4000 cm^{-1}.

3.2.4.4. Nuclear magnetic resonance (^{1}H NMR) spectroscopy

^{1}H NMR spectra were scanned using a JEOL JNM-LA 400 FT NMR spectrometer (400 MHz) (Tokyo, Japan). H-chemical shifts were given relative to external tetramethylsilane (TMS). The spectra were performed in DMSO and D_2O.

3.2.5. Dissolution studies

The dissolution behavior of the AMB, its inclusion complex or a physical mixture was examined in water according to the USP type II dissolution method using an Erweka equipment, model DT-06, Germany. An amount of each sample equivalent to 20 mg of AMB was introduced into 250 ml

of water maintained at 37 °C and stirred at 100 rpm. At appropriate intervals, 2 ml was withdrawn and filtered using a millipore filter (0.45 μm), diluted with 0.1 NaOH and measured at 252 nm spectrophotometrically. Two ml of water was added each time to maintain the volume of the dissolution medium constant.

3.2.6. In-vivo study

The hypnotic action of AMB was evaluated using mice. Ten mice, average weight 20 g, were used throughout the study. They were housed in aluminium cages at a constant temperature and humidity under a dark-light cycle of about 12 h for several days. They had free access to standard granulated food and tap water. The animals were randomly divided into two groups of five mice each. AMB solution with or without CD was given to the mice at the dose of 1.6 mg/animal using a stomach tube. The onset of action (the time until the onset of loss of righting reflex after drug adminstration) and the duration of hypnosis (the time until recovery from the loss of righting reflex) were measured using a stop watch.

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