Preparation and Properties of Fluorescent Labels Coumarin-6/HP-β-CD Complex by Nasal Drug Delivery

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Abstract. In order to improve the solubility and dissolution of coumarin-6, coumarin-6/HP-β-CD complex was prepared by freeze-drying. Phase solubility, UV-vis spectroscopy and fourier transformation infrared spectrum results showed that as the concentration of HP-β-CD increased, the soluble coumarin-6 in solution increased and it can be encapsulated to the cavity of HP-β-CD. Then the dissolution result showed that the complex of HP-β-CD can effectively increase the dissolution rate of coumarin-6.

1. Introduction
Coumarins are widely found in the secondary metabolites of higher plants and have strong aromatic smell. The coumarin and its derivatives are mostly in the form of single coumarin and a few in the form of bimolecular or trimolecular polymers. It can be regarded as a kind of lactone compound. They have the functions of anti-tumor, anti-oxidation, anti-HIV, anti-coagulation, anti-hypertension, anti-arrhythmia, anti-bacterial, anti-cough, anti-asthma, anti-inflammatory and analgesic. They have high medicinal value, and they are also hot fields in the research and development of health care products. But their application in clinic is limited because of its poor solubility in aqueous solution. Coumarins are well-known laser dyes in the blue-green region, and are usually used in the study of biological pathways. Coumarin-6 is a member of 7-aminocoumarins. Coumarin-6 is a hydrophobic drug with small molecular weight, pyranopentose structure and low solubility in water. It is a traditional fluorescence dye for in vitro and in vivo monitoring [1]. It can be incorporated in the drug delivery system and used for nasal administration of drugs to track the fluorescent intensity in various parts of the brain [2]. And it can be used as the model compound that incorporated into the drug loading system [3]. Coumarin-6 has a role as fluorochrome with high laser conversion rate, relatively stable performance to perform optical transmission and luminescence measurements [4-5]. And the photophysical properties of coumarin-6 were different in non-polar and other solvents [6]. Coumarin-6 encapsulated polystyrene nanospheres conjugated with peanut agglutinin (PNA) and poly (N-vinylacetamide) (PNVA) could be developed as a safe diagnostic agent for colonoscopy applications [7]. It can be used to prepare luminescent collectors based on epoxy resins [8]. By forming supramolecular complex with the main macromolecules, the solubility of supramolecular complex in aqueous solution can be improved obviously.
Cyclodextrins (CD) are cyclic oligosaccharides that have a hydrophilic surface with a hydrophobic central cavity, favoring the formation of complex with low polarity drugs [9]. The CD with the most widely application in the pharmaceutical field is the β-CD for an easy drug complexation at low costs. 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) is a kind of chemically modified derivatives of CD and has been widely used on account of its better solubility and increased complex when compared to the natural ones.

2. Materials

2.1. Chemicals and reagents

Coumarin-6 (purity of 98 %) and HP-β-CD (molecular weight of 1540) were purchased from Sigma-Aldrich. Absolute ethyl alcohol was supplied by Tianjin chemical reagent supply and marketing company. All water mentioned was deionized water, other reagents used were standard analytical pure.

2.2. Instruments

Digital Display Constant Temperature Water Bath was purchased from Jiangsu jintan jincheng guosheng experimental instrument factory. Electronic balance was provided by Ohaus instrument co., LTD. UV2401 ultraviolet spectrophotometer was purchased from Shimadzu, Japan. CL-4B magnetic stirrer was supplied by Gongyi yuhua instrument co., LTD. IR Fourier Spectrometer was purchased from Bruker, Germany. Vacuum freeze dryer was supplied by Beijing boyikang experimental instrument co., LTD.

3. Methods

3.1. Drawing the standard curve of coumarin-6

10 mg coumarin-6 was accurately weighed and dissolved in anhydrous ethanol, then fixed volume in 50 mL flask with anhydrous ethanol, the mother liquor of 0.200 mg/mL coumarin-6 was obtained. 10 mL of solution was removed from the mother liquor and placed in a 50 mL flask then fixed volume with anhydrous ethanol, and the intermediate solution was obtained. Then 0.025, 0.125, 0.25, 0.5, 1, 2, and 4 mL of solution were extracted from intermediate solution to 10 mL flask respectively. Ultraviolet spectrophotometer was used to determine the absorbance at the wavelength of 462 nm with anhydrous ethanol as the reference solution. The standard curve was drawn with the concentration of coumarin-6 (C) as the abscissa and absorbance (A) as the ordinate, and the standard curve equation was obtained.

3.2. Phase solubility

The concentration of HP-β-CD solution was 3.0 × 10^{-3}, 6.0 × 10^{-3}, 9.0 × 10^{-3}, 12.0 × 10^{-3}, 15.0 × 10^{-3}, 18.0 × 10^{-3}, 30 × 10^{-3}, and 40 × 10^{-3} mol/L. Excessive coumarin-6 was added respectively and then stirred for 8 h at 800 rpm. After reaching the equilibrium of solution, took the supernatant and filtered it with microporous membrane with diameter 0.45 microns. The equilibrium phase solubility curve was drawn with the molar concentration of HP-β-CD solution as the abscissa and the molar concentration of coumarin-6 as the ordinate.

3.3. UV-vis spectroscopy of coumarin-6/HP-β-CD complex

20 mL HP-β-CD solution with concentrations of 2.0, 4.0, 8.0 and 10.0mmol/L was prepared respectively, and 0.007g coumarin-6 was added respectively. Ultraviolet spectroscopy scanning was performed in the range of 200 ~ 600 nm at room temperature.

3.4. Preparation of coumarin-6/HP-β-CD complex

The mole ratio of coumarin-6 and HP-β-CD was weighed at 1:1. HP-β-CD and rotor were put in a beaker on a magnetic stirring, water was added and then stirred to dissolve. Coumarin-6 was dissolved
in anhydrous ethanol. Coumarin-6 ethanol solution was added to HP-β-CD with a syringe then mixed them under the speed of 800 rpm for 8 h on a magnetic stirring. Micro porous membrane with diameter 0.45 microns was used to filter, filter liquor was froze to solid in -20°C refrigerator, then it was freeze-dried with vacuum freezing dryer to obtain coumarin-6/HP-β-CD complex.

3.5. Determination of Inclusion rate

The mass of coumarin-6/HP-β-CD complex (M) was weighed and dissolved in a 10mL flask and fixed volume with absolute ethyl alcohol. The absorbance at 462 nm was determined by ultraviolet spectrophotometer, and then the mass of coumarin-6 (m) was quantified according to the standard curve.

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\text{Inclusion rate (\%)} = \frac{m}{M} \times 100\%
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3.6. Fourier transformation infrared spectrum (FTIR) of coumarin-6/HP-β-CD complex

The FTIR of coumarin-6, HP-β-CD, physical mixture and coumarin-6/HP-β-CD complex were measured respectively by KBr tablet method in the wavelength range from 4000 cm\(^{-1}\) to 500 cm\(^{-1}\), and the characteristic absorption peaks were compared.

3.7. Dissolution rate of coumarin-6/HP-β-CD complex

With reference to dissolution determination method, the release medium dissolution with pH 6.4 phosphate buffer solution (PBS) which pH environment close to the nasal cavity was used in the improved determination. 60 mL degassing process PBS which had warmed up to 34 °C was added in two dissolution beaker respectively. Then they were put on magnetic stirring with speed of 50 rpm, the temperature was kept about 34.0 °C. 1 g coumarin-6/HP-β-CD complex (equivalent to about 1.3 mg of coumarin-6) and 1.3 mg of coumarin-6 were added respectively in dialysis membrane, and then placed them in two dissolution beakers. After 15, 30, 45, 60, 90, 120, 180, 240min sampled respectively, then same volume of PBS which has been preheated to 34°C was added. Micro porous membrane with diameter 0.45 microns were used to filter, ultraviolet spectrophotometer was used to determine. The dissolution curve in vitro was obtained with time as the abscissa and coumarin-6 concentration as the ordinate.

4. Results and Discussion

4.1. Phase solubility

Phase solubility curve of coumarin-6 was shown in Figure 1. The results showed that as the concentration of HP-β-CD increased, the solubility of coumarin-6 in linearly increased. According to the study of Higuchi\(^{[10]}\), the phase dissolution curve conforms to the A\(_L\) type, indicating the formation of inclusion compound with molar ratio of 1:1, and the two have good interaction.

4.2. UV-vis spectroscopy of coumarin-6/HP-β-CD complex

The ultraviolet absorption of coumarin-6 with the gradual increase of the concentration of HP-β-CD (range from 2.0 to 10.0 mmol/L) was shown in Figure 2. The results showed that coumarin-6 presented the maximum absorption at 462 nm, and the absorbance of coumarin-6 gradually decreased at 462 nm when the concentration of HP-β-CD increased successively from 2.0 to the 10.0 mmol/L. This indicated that coumarin-6 was encapsulated to the cavity of HP-β-CD. Therefore, decrease in absorbance indicated that coumarin-6/HP-β-CD complex was formed.
4.3. FTIR of coumarin-6/HP-β-CD complex
The FTIR of coumarin-6, HP-β-CD, physical mixtures and coumarin-6/HP-β-CD complex were shown in Figure 3. The results showed that coumarin-6 had strong absorption peaks at 1448, 1346, 935, 820 and 756 cm\(^{-1}\), which were characteristic absorption peaks of coumarin-6. The characteristic absorption peaks of HP-β-CD were 3409 cm\(^{-1}\) and 2929 cm\(^{-1}\), respectively. The absorption peaks of the physical mixture matched those of coumarin-6 and HP-β-CD, respectively. This indicating that there was no interaction between coumarin-6 and HP-β-CD. FTIR of coumarin-6/HP-β-CD complex showed wide absorption at 3401 cm\(^{-1}\), while small strong absorption at 1448, 1346 and 935 cm\(^{-1}\), and the sharp absorption peaks of coumarin-6 at 756 and 820 cm\(^{-1}\) were disappeared. Based on the analysis of the above results, coumarin-6 may be encapsulated in the cavity of HP-β-CD molecule to form the complex.

4.4. Dissolution rate of coumarin-6/HP-β-CD complex
In dissolution rate of coumarin-6/HP-β-CD complex and coumarin-6 were shown in the Figure 4. The dissolution rate of coumarin-6 in the PBS that simulates the nasal environment was very low, while the dissolution rate of the coumarin-6/HP-β-CD complex increased over time. And the dissolution rate
was much higher than coumarin-6. The results showed that the complex of HP-β-CD can effectively increase the dissolution rate of coumarin-6.

Figure 3. FTIR of coumarin-6, HP-β-CD, physical mixtures and coumarin-6/HP-β-CD complex

Figure 4. In vitro release rates of coumarin-6/HP-β-CD complex compound and coumarin-6
5. Conclusion
The results above showed that after the complex of HP-β-CD, the solubility and in dissolution rate of coumarin-6 were significantly increased, which could provide a basis for clinical development and utilization of coumarin-6 in nasal drug delivery system.

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References
[1] Xiaoqing Miao, Ye Li, Xueqing Wang, Simon Ming-Yuen Lee, Ying Zheng, Transport Mechanism of Coumarin 6 Nanocrystals with Two Particle Sizes in MDCKII Monolayer and Larval Zebrafish, ACS Applied Materials & Interfaces, 2016(8):12620-12630.
[2] Yogesh K. Katare, Ritesh P. Daya, Christal Sookram Gray, Roger E. Luckham, Jayant Bhandari, Abhay S. Chauhan, Ram K. Mishra, Brain Targeting of a Water Insoluble Antipsychotic Drug Haloperidol via the Intranasal Route Using PAMAM Dendrimer, Molecular Pharmaceutics, 2015(12):3380-3388.
[3] Jinyu Li, Guoxin Tan, Bingchao Cheng, Dandan Liu, Weisan Pan, Transport mechanism of chitosan-N-acetylcysteine, chitosan oligosaccharides or carboxymethyl chitosan decorated coumarin-6 loaded nanostructured lipid carriers across the rabbit ocular, European Journal of Pharmaceutics and Biopharmaceutics, 2017(120):89-97.
[4] Monica Enculescu, Growth and optical characteristics of coumarin 6 doped potassium hydrogen phthalate (KAP) crystals, Optical Materials, 2009(32):281-285.
[5] S.M.Z. Al-Kindy, S.A. El-Sherbini, M.H. Abdel-Kader, UV-visible absorption and fluorescence characteristics of the luminescent label coumarin-6-sulphonyl chloride in homogeneous and micellar solutions, Analytica Chimica Acta, 1994(285):329-333.
[6] Ashis Kumar Satpati, Manoj Kumbhakar, Dilip Kumar Maity, Haridas Pal, Photophysical investigations of the solvent polarity effect on the properties of coumarin-6 dye, Chemical Physics Letters, 2005(407):114-118.
[7] Sakuma S, Kumagai H, Shimosato M, Kitamura T, Mohri K, Ikejima T, Hiwatari K, Koike S, Tobita E, McClure R, Gore JC, Pham W. M, etc. Toxicity studies of coumarin 6-encapsulated polystyrene nanospheres conjugated with peanut agglutinin and poly (N-vinylacetamide) as a colonoscopie imaging agent in rats. Nanomedicine. 2015 Jul; 11(5):1227-36.
[8] Matteo Sottile, Giovanni Tomei, Silvia Borsacchi, Francesca Martini, Marco Geppi, Giacomo Ruggeri, Andrea Pucci. Epoxy resin doped with Coumarin 6: Example of accessible luminescent collectors, European Polymer Journal, 2017(89): 23-33.
[9] Thorsteinn Loftsson, Dominique Duchêne, Cyclodextrins and their pharmaceutical applications, International Journal of Pharmaceutics, 2007 (329):1-11.
[10] Higuchi, T. Phase solubility techniques. Adv.anal.chem.instr. 1965; 4: 117-212.