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A novel multi-tiered experimental approach unfolding the mechanisms behind cyclodextrin-vitamin inclusion complexes for enhanced vitamin solubility and stability

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ABSTRACT

This study was conducted to provide a mechanistic account for understanding the synthesis, characterization and solubility phenomena of vitamin complexes with cyclodextrins (CD) for enhanced solubility and stability employing experimental and *in silico* molecular modeling strategies. New geometric, molecular and energetic analyses were pursued to explicate experimentally derived cholecalciferol complexes. Various CD molecules (α-, β-, γ-, and hydroxypropyl β-) were complexed with three vitamins: cholecalciferol, ascorbic acid and α-tocopherol. The Inclusion Efficiency (IE%) was computed for each CD-vitamin complex. The highest IE% achieved for a cholecalciferol complex was for 'βCDD$_3$-8', after utilizing a unique CD:cholecalciferol molar synthesis ratio of 2.5:1, never before reported as successful. 2HPβCD-cholecalciferol, γCD-cholecalciferol and α-tocopherol inclusion complexes (IC's) reached maximal IE% with a CD:vitamin molar ratio of 5:1. The results demonstrate that IE%, thermal stability, concentration, carrier solubility, molecular mechanics and intended release profile are key factors to consider when synthesizing vitamin-CD complexes. Phase-solubility data provided insights into the design of formulations with IC's that may provide analogous oral vitamin release profiles even when hydrophobic and hydrophilic vitamins are co-incorporated. Static lattice atomistic simulations were able to validate experimentally derived cholecalciferol IE phenomena and are invaluable parameters when approaching formulation strategies using CD's for improved solubility and efficacy of vitamins.

1. Introduction

Vitamins are sensitive biologically active macromolecules that function as important co-factors in an array of vital cellular processes and are not exempt from pharmaceutical challenges. Although providing exciting opportunities for alternative and complementary medical advances when used in targeted doses, many are highly susceptible to degradation, possess poor bioavailability, and lack the necessary robustness when consumed in their pure vitamin forms (Dreassi et al., 2010; Gonnet et al., 2010; Benshitrit et al., 2012; Saldanha and Tollefsbol, 2012). For example, α-tocopherol is problematic to formulate due to an intrinsic instability to UV light and heat (Cassano et al., 2009). Vitamin C is an antioxidant with a wide range of health purporting effects however most activity is lost during processing and long-term storage due to exposure to environmental factors and degradation (Alishahi et al., 2011; Paneva et al., 2011). Vitamin A and its precursors are known to degrade rapidly in aqueous solution with an accompanied loss of activity, are susceptible to degradation during high temperature processing, and are also known for low solubility and dispersibility in aqueous medium (Loveday and Singh, 2008; Zaibunnisa et al., 2011a,b; Sauvant et al., 2012). Vitamins D, E, and K are a group of essential lipophilic vitamins documented to have low solubility in aqueous media and pose significant formulation challenges (Ramalho Merce et al., 2009). Cholecalciferol has many health benefits but when dosed orally often fails to achieve the intended and rapid clinical response due to low aqueous solubility and reliability on food and other co-factors for absorption (Moyad, 2009; Braithwaite et al., 2013). In lieu of these formulation challenges, vitamins are being increasingly modified to enhance their potency and to manipulate their physicochemical properties in favor of improved complementary treatment approaches to disease (Schwartz, 2009; Rassnick et al., 2011). Delivery systems impart improvements in solubility and stability

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to actives whilst achieving successful targeted release profiles (Benshitrit et al., 2012).

Cyclodextrin (CD) molecules are documented as valuable complexing agents for use with drugs, food constituents and nutraceuticals (Giordano et al., 2001; Del Valle, 2004; Yang et al., 2009; Racz et al., 2012). The pharmaceutical, cosmetic and food industry have long since researched and utilized these compounds as a means to modify, manipulate and alter the physicochemical and biological properties of actives in an attempt to advance scientific research and development (Polyakov et al., 2004). The inclusion complex (IC) formed between a CD and a bioactive molecule may provide physical isolation of incompatible compounds in a multi-component formulation or afford the controlled release of a bioactive. CD molecules have also been proven to be highly effective in providing protection against heat, evaporation, oxidation and degradation of sensitive bioactives (Terekhova and Kulikov, 2002; Astray et al., 2009). Thus, CD molecules are able to assist with overcoming many of the challenges mentioned above to improve bioavailability, solubility, penetration, stability, and to reduce membrane irritant effects and interactions between bioactives (Del Valle, 2004; Zhenming et al., 2003). CD-bioactive interactions are complex and may be inclusion based or non-inclusion based involving the binding of a bioactive to the edge of a CD molecule, partial infiltration into the glucopyranose ring, formation of aggregates, or stable multi-drug supersaturated systems (Loftsson et al., 2007; Dreassi et al., 2010; Kurkov and Loftsson, 2013). The complexation of all-*trans*-retinoic-acid with hydroxypropyl β-cyclodextrin increased the vitamin's aqueous solubility more than 10,000 times (Sauvant et al., 2012). Despite βCDs having low aqueous solubility, they are still the most commonly used, with studies showing the ability of these complexes to considerably increase the dissolution rate of poorly water soluble compounds (Dua et al., 2011; Kurkov and Loftsson, 2013). A high assimilation potential was also shown in an *in vitro* model of the intestinal membranes when vitamins such as carotenoids were delivered as IC's with βCD (Fernandez-Garcia et al., 2010). Other researchers used a nanoparticle dispersion containing a modified βCD-drug complex to release tacrolimus at a sustained rate for an improved side-effect profile (Gao et al., 2012). γCD-complexes have limited aqueous solubility due to aggregate formation, however in the presence of intestinal bile acids there has been a substantial improvement in bioavailability reported in some studies (Haiyee et al., 2009; Uekaji et al., 2012). Hydroxypropyl-β-CD is a modified CD molecule with high water-solubilizing power. Methylated βCD was able to adsorb high quantities of antimicrobials when prostheses were functionalized with this carrier, showing promise as a versatile treatment of vascular prostheses infections (Blanchemain et al., 2011). Methylated β-CD has been successfully used as a component of novel carrier complexes for various vitamins (Fathi-Azarbayjani et al., 2010).

Despite studies investigating the complexation of individual vitamins with specific CD's, there are limited reports on the mechanisms and synthesis of complexes from varied molar synthesis ratios of vitamins and CD's and resultant inclusion efficiency (IE) values. Simultaneous synthesis of different vitamin: CD complexes, followed by characterization, and molecular mechanics (MM+) simulations of selected complexes, may provide invaluable insights into optimal combinations. Only a few researchers have undertaken computational molecular mechanics simulations of vitamins and cyclodextrins (Mallem et al., 2012), and even fewer have conducted comprehensive experimentation of complex synthesis in conjunction with *in silico* molecular mechanics simulations thereof. There has been recent interest in cholecalciferol and its active metabolites and methods to improve delivery (Li et al., 2013), yet the benefits of CD technology have yet to be optimized for inclusion in vitamin formulations on the market. CD insertion in multivitamins may provide solutions to minimizing deleterious interactions, enhancing bioavailability and stability, and even enabling a customized release strategy of individual constituents. Complexation of CD's with vitamins is also hypothesized as a method to modify the aqueous release of vitamins of diverse physical properties. The current study provides a mechanistic account for understanding the synthesis, characterization and solubility phenomena of various vitamin complexes, and rationalizes the efficiency of formation of different CD complexes with 3 different vitamins (cholecalciferol, α-tocopherol and ascorbic acid) as candidates of commercial multivitamin products. Additionally, this study provides the first complete geometric, molecular and energetic mechanistic analysis of vitamin D_3 (cholecalciferol) with key cyclodextrin analogues. The study in particular forms the basis of an approach for administering three vitamins with varied physical properties in one formulation while obtaining a concerted release profile for a "maximal optimal synergistic nutrotherapeutic paradigm" with vitamin D_3 being the primary therapeutic agent.

2. Materials and methods

2.1. Materials

Cholecalciferol 1000 IU (vitamin D), α-tocopherol (vitamin E), ascorbic acid (vitamin C) and various cyclodextrin (CD) molecules (α-, β-, γ- and 2-HPβ) were purchased from Sigma Aldrich (St. Louise, MO, USA). Organic solvents utilized included analytical grade ethanol, methanol, acetone and ethyl acetate (Sigma Aldrich, St Louise, MO, USA).

2.2. Synthesis of the CD-vitamin inclusion complexes

CD was added to the bioactive vitamins (V) in varying molar ratios as per a modified co-precipitation method. A customized method utilizing additional molar ratios of CD:vitamin and extended mixing times was employed to investigate complexation efficiency. The following CD:vitamin ratios were investigated where molar ratios of CD:V varied from 10:1; 5:1 and 2.5:1.

- CD (0.2 mmol):V (0.02 mmol)-designated CD-vitamin-2 (10:1)
- CD (0.2 mmol):V (0.04 mmol)-designated CD-vitamin-4 (5:1)
- CD (0.2 mmol):V (0.08 mmol)-designated CD-vitamin-8 (2.5:1)

Briefly, CD molecules and vitamins were weighed before addition of solvent. Stock solvent solutions were prepared as follows: ethanol ($50\%^{v}/_{v}$) for cholecalciferol, acetone (100%) for α-tocopherol and deionized water for ascorbic acid. In certain instances the vitamin was first dissolved in the organic solvent separately whilst the CD was dissolved in water and then the solutions were combined (α-tocopherol). Each of the CD-vitamin solutions were stirred overnight at room temperature for pre-determined time periods (16 h, 18 h, 20 h, or 40 h) to test whether mixing time influenced complexation. After mixing, the CD-vitamin mixtures were poured onto petri dishes to allow solvent evaporation at room temperature for 48 h. The resultant solid precipitate powder was collected for further analysis. The precipitate contained the respective CD-vitamin complexes and uncomplexed 'free' vitamin. Ethanol (20 mL) was used as the solvent to remove free vitamin from the following complexes: αCD-cholecalciferol, βCD-cholecalciferol, γCD-cholecalciferol and βCD-vitamin C. Acetone (20 mL) was used to remove 'free' (non-complexed) α-tocopherol from βCD-α-tocopherol complexes. Ethylacetate (20 mL) was used to remove uncomplexed cholecalciferol from 2HPβ-cyclodextrin-cholecalciferol complexes.

2.3. Preparation of CD-vitamin physical mixtures

Each vitamin powder (cholecalciferol, α-tocopherol, or ascorbic acid) was physically blended with the respective CD in an equimolar ratio as to prepare the IC's. Dry native CD (0.2 mmol, α-, β- or γ-) was added to a petri dish containing 0.02, 0.04, or 0.08 mmol of the respective vitamin. A spatula was used to mechanically blend the 2