Hydrolysis Behavior of Prednisolone 21-Hemisuccinate/β-Cyclodextrin Amide Conjugate: Involvement of Intramolecular Catalysis of Amide Group in Drug Release

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Prednisolone 21-hemisuccinate/β-cyclodextrin (β-CyD) amide conjugate was prepared by binding prednisolone 21-hemisuccinate covalently to the amino group of mono(6-deoxy-6-amino)-β-CyD through amide linkage. Prednisolone 21-hemisuccinate was intramolecularly transformed to prednisolone 17-hemisuccinate, and the parent drug, prednisolone, was slowly released from the 21-hemisuccinate with a half life of 69 h in pH 7.0 at 37 °C; the drug release at 25 °C was less than 10% for 48 h. In sharp contrast, the hydrolysis of prednisolone 21-hemisuccinate/β-CyD amide conjugate was significantly faster (half life of 6.50 min at 25 °C) and gave prednisolone and mono(6-deoxy-6-succimino)-β-CyD as products. The hydrolysis of the β-CyD amide conjugate was subject to a specific-base catalysis in the alkaline region. The rapid hydrolysis of the conjugate can be ascribed to the involvement of an intramolecular nucleophilic catalysis of the amide group in the reaction. The succinic acid, bound to a drug through ester linkage at one carboxylic group and bound to a pro-moiety through amide linkage at another carboxylic group, may be useful as a spacer for construction of the immediate release type prodrugs of CyDs.

Key words cyclodextrin; prednisolone 21-hemisuccinate/β-cyclodextrin amide conjugate; hydrolysis

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6—8 glucose units through α-1,4 glucosidic bonds and have been utilized for improvement of certain properties of drugs such as solubility, stability and bioavailability, *etc.*, through the formation of inclusion complexes.[1—4] However, CyD complexes seem to be unsuitable for drug targeting or control of the pharmacokinetic properties of distribution and elimination, because of a fast dissociation of the complex into each component in body fluids.[5] One method to circumvent the dissociation is to bind a drug covalently to CyDs. A number of CyD derivatives have been prepared for purposes such as the construction of enzyme models, chiral separators or recognizing agents.[6—8] However, applications of drug/CyD conjugates to drug targeting or control of pharmacokinetic properties are very scarce.[9] In previous studies, we prepared CyD conjugates where one of the primary hydroxyl groups of α-, β- and γ-CyDs was substituted by an anti-inflammatory drug, 4-biphenylylacetic acid, through an ester- or amide-linkage.[10—12] These ester conjugates released the drug site-specifically in cecal and colonic contents of rats after fermentation of CyD rings to small saccharides, suggesting that CyD conjugations are useful for construction of colon-specific drug delivery systems or delayed-release systems in oral preparations. In this study, we prepared a prednisolone 21-hemisuccinate/β-CyD conjugate, in which one of the primary hydroxyl groups of β-CyD was substituted by the drug through an amide bond, and its *in vitro* drug release behavior was kinetically investigated.

Experimental

Materials β-CyD was supplied by Japan Maize Co. (Tokyo, Japan) and prednisolone 21-hemisuccinate was donated by Ono Pharmaceutical Co. (Osaka, Japan). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

Analytical Apparatus ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were taken on a JEOL GX-400 or A-500 spectrometer (Tokyo) at 25 °C. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-DX 303 mass spectrometer (Tokyo) in a negative mode using a matrix of diethanolamine/dimethyl sulfoxide (DMSO).

Preparation of Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate Mono(6-deoxy-6-amino)-β-CyD was obtained in three steps from the parent β-CyD (Chart 1),[13] *i.e.*, one of the primary alcohols of β-CyD was tosylated using *p*-toluenesulfonyl chloride, followed by conversion to mono(6-azide-6-deoxy)-β-CyD using sodium azide and, in turn, reduction to the monoamino-β-CyD which was purified by gel chromatography (CM-Sephadex C-25 (NH_2 form), 2×25 cm, Pharmacia Biotech, Sweden) with aqueous NH_4HCO_3 solution. To prednisolone 21-hemisuccinate (0.195 g, 0.42 mmol)/anhydrous ethyl acetate (100 ml) was added hydoxysuccimide (0.051 g, 0.44 mmol) and 1,3-dicyclohexylcarbodiimide (DCC) (0.091 g, 0.44 mmol), and the mixture was stirred at room temperature (about 25 °C) for 30 h. The reaction was monitored by thin layer chromatography (TLC) using normal-phase plates precoated with silica gel $60F_{254}$ obtained from Merck (Darmstadt, Germany) and an eluent of chloroform/methanol=9 : 1 (indicator: UV at 254 nm). After the filtration, the solution was dried under reduced pressure. The monoamino-β-CyD (0.57 g, 0.5 mmol) was added to the residue/DMF (5 ml), and the mixture was stirred at room temperature for 16 h. The reaction solution was concentrated under reduced pressure, and then acetone (100 ml) was added. The precipitate was collected by filtration, washed thoroughly with acetone, and purified by preparative HPLC using YMC SH-363-5 ODS column (30×250 mm, Kyoto, Japan) and a mobile phase of methanol/0.05 M acetic acid (1 : 1 v/v). Acetic acid was added to the mobile phase, because the conjugate was stable in acidic regions. However, because the prednisolone 21-hemisuccinate/β-CyD amide conjugate was extremely labile in water as described later, the conjugate contained small amounts of the monoamino-β-CyD (<5%) which was formed during the column separation, although the parent drug, prednisolone, was completely separated by washing the conjugate with acetone or ethanol. Yield (498 mg, 63.2%), R_f=0.58 (silica gel TLC plate (Merck F_{254}), ethyl acetate/2-propanol/water=7 : 7 : 5 v/v), FAB mass $[M-H]^-$ *m/z* 1575, ^{1}H-NMR (500 MHz, DMSO-d_6); δ 7.35—7.33 (d, 1H, steroid 1-H), 6.16—6.14 (d, 1H, steroid 2-H), 5.90 (s, 1H, steroid 4-H), 5.06—4.72 (m, 9H, steroid 21-H, CyD 1-H), 4.26 (s, 1H, steroid 11-H), 4.04—3.18 (overlaps with HOD m, CyD 2, 3, 4, 5, 6-H), 2.62—2.26 (m, overlaps with DMSO-d_6, succinyl–CH_2–CH_2–, steroid 6β, 7β, 8, 16β-H), 2.00—1.78 (m, 3H, steroid 12α, 14β, 15α,-H), 1.40—1.26 (m, 3H, steroid 6α, 12β-H), 1.34 (s, 3H, steroid 19-CH_3), 1.12-1.11 (dd, 1H, steroid 15β-H), 0.98—0.95 (dd, 1H, steroid 7α-H), 0.87—0.83 (d, 1H, steroid 9-H), 0.73 (s, 3H, steroid 18-CH_3).

Preparation of Mono(6-deoxy-6-succimino)-β-CyD The prednisolone 21-hemisuccinate/β-CyD amide conjugate (30 mg) was incubated at pH 9.0 and 25 °C for 1 h. The water was evaporated under reduced pressure and the

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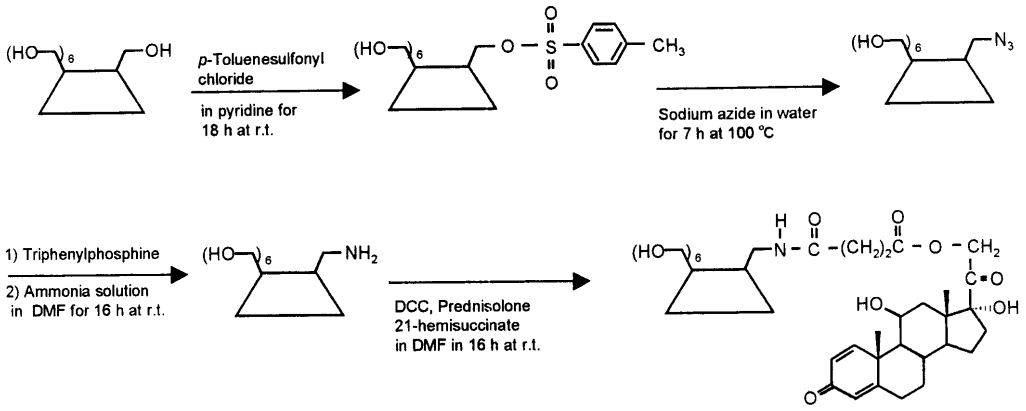

Chart 1. Preparation of Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate

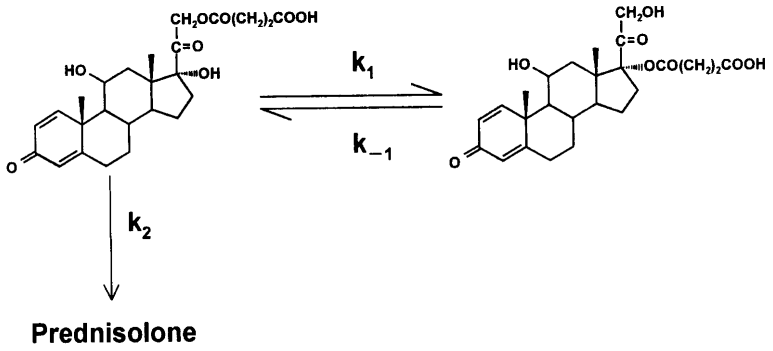

Chart 2. Hydrolysis Mechanism of Prednisolone 21-Hemisuccinate

residue was subjected to ion exchange chromatography for separation of the succimidyl β-CyD : Amberlite IRC50 (acid form, 2×25 cm, Organo Co., Japan) with water. Yield (18 mg, 66.6%), R_f=0.38 (silica gel TLC plate (Merck F_{254}), ethyl acetate/2-propanol/water=7 : 7 : 5 v/v), FAB mass $[M-H]^-$ *m/z* 1214, ^{1}H-NMR (500 MHz, DMSO-d_6); δ 4.91—4.69 (m, 7H, CyD 1H), 3.85—3.53 (m, 28H, CyD 3, 5, 6-H), 3.44—3.04 (m, 18H, CyD 2, 4-H), 2.61—2.40 (m, overlaps with DMSO-d_6, succinyl–CH_2–CH_2–). ^{13}C-NMR (DMSO-d_6); δ 178.3 (succinyl C=O), 101.8 (CyD C1), 81.2 (CyD C4), 72.8—72.0 (CyD C2, 3, 5), 59.8 (CyD C6), 28.5 (succinyl–CH_2–CH_2–).

Hydrolysis of Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate The hydrolysis of the prednisolone/β-CyD amide conjugate was performed by adding the conjugate/DMF solution (10.0 μl, 1.35×10^{-3} M) into phosphate buffers (5.0 ml, pH 2—7, ionic strength=0.2) at 25 °C. At timed intervals, an aliquot (20.0 μl) was subjected to HPLC analysis for prednisolone and the prednisolone/β-CyD amide conjugate under the following conditions : A Hitachi 655A pump with a L-4000 variable wavelength ultraviolet detector (Tokyo), a YMC A-303 ODS column (5 μm, 4.6×250 mm, Kyoto), an eluent of methanol/water (1 : 1 v/v) and a detection of 254 nm for prednisolone and the prednisolone/β-CyD conjugate, a flow rate of 1.0 ml/min. The succimidyl β-CyD conjugate was monitored by TLC using normal-phase plates precoated with silica gel $60F_{254}$ obtained from Merck (Darmstadt, Germany) and an eluent of ethyl acetate/2-propanol/water=7 : 7 : 5 (indicator : *p*-anisaldehyde). The hydrolysis of prednisolone 21-hemisuccinate was conducted under the same experimental conditions, except for the reaction temperature (37 °C) because of the slow hydrolysis at 25 °C.

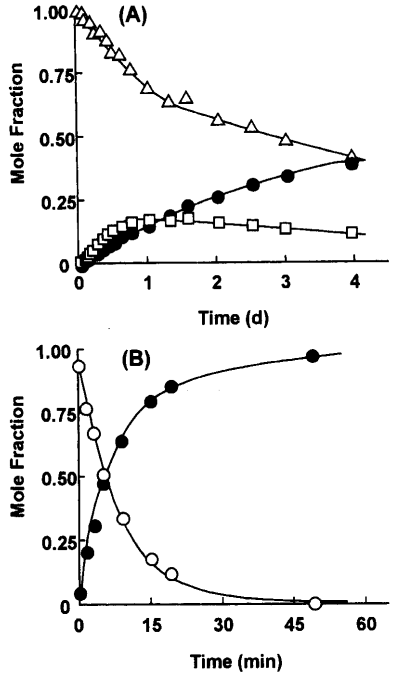

Fig. 1. Time Courses for Hydrolysis of Prednisolone 21-Hemisuccinate (A, at 37 °C) and Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate (B, at 25 °C) in pH 7.0 Phosphate Buffer

△, prednisolone 21-hemisuccinate; □, prednisolone 17-hemisuccinate; ●, prednisolone; ○, amide conjugate.

Results and Discussion

Figures 1A and 1B show reaction profiles for the hydrolysis of prednisolone 21-hemisuccinate at 37 °C and the prednisolone 21-hemisuccinate/β-CyD amide conjugate at 25 °C, respectively, in pH 7.0 phosphate buffer. Prednisolone 21-hemisuccinate transformed intramolecularly to prednisolone 17-hemisuccinate and slowly released the parent drug, prednisolone. The hydrolysis profiles of prednisolone 21-hemisuccinate were analyzed according to the reaction mechanism shown in Chart 2, where the 21-hemisuccinate is in equilibrium with the 17-hemisuccinate and prednisolone is released from the 21-hemisuccinate. The experimental data fitted closely to the theoretical curves. On the other hand, the

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Table 1. First-order Rate Constants (h^{-1}) for Hydrolyses of Prednisolone 21-Hemisuccinate and Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate in Phosphate Buffer (pH 7.0, I=0.2)

Compound	k_1	k_{-1}	k_2
Prednisolone 21-hemisuccinate (at 37 °C)	0.031	0.147	0.010
β-CyD amide conjugate (at 25 °C)	—	—	6.40[a]

a) A first-order rate constant of prednisolone release from the conjugate.

hydrolysis profiles did not fit the reaction scheme where prednisolone is released from both 21-and 17-hemisuccinates. Therefore, the rate constants (k_1, k_{-1} and k_2) were obtained from analysis of the reaction profiles (Fig. 1A) according to Chart 2, using a nonlinear least-squares method,[14] as listed in Table 1. The backward reaction (k_{-1}) was about 5 times the forward reaction (k_1) in the equilibrium of the acyl migration. The time required to reach 50% release of prednisolone was 69 h at pH 7.0, 37 °C, whereas at 25 °C the drug release was less than 10% for 48 h. These results agreed with those reported by Anderson and Taphouse.[15] On the other hand, the hydrolysis of prednisolone 21-hemisuccinate/β-CyD amide conjugate was significantly faster than that of prednisolone 21-hemisuccinate (Fig. 1B). The β-CyD amide conjugate was hydrolyzed according to first-order kinetics and quantitatively released prednisolone with the concomitant decrease in concentration of the conjugate with a rate constant of 6.40 h^{-1} (half life of 6.50 min, Table 1) at 25 °C. The apparent release rate of prednisolone from the β-CyD amide conjugate was faster by a factor of 640 than that from the 21-hemisuccinate, even when the rates were compared at different temperatures. Prednisolone 21-hemisuccinate gave prednisolone and succinic acid as hydrolysis products under the experimental conditions. When the hydrolysis of the β-CyD amide conjugate was monitored by TLC, a new additional spot emerged at R_f value of 0.38 (silica gel plate, ethyl acetate/2-propanol/water=7:7:5). This new product was separated by ion exchange chromatography (water/DMF=1:1 v/v) and analyzed by mass and NMR spectroscopies for its structure determination. The new product gave a parent ion at m/z 1214 in the FAB mass spectrum (Fig. 2), indicating a lack of the steroid skeleton. The ^{13}C-NMR spectrum of the product gave two new signals at 178.3 and 28.5 ppm corresponding to carbonyl and methylene carbons, respectively, together with a set of β-CyD signals (101.8—59.8 ppm). In the ^{1}H-NMR spectrum, the product gave a new peak at 2.61—2.40 ppm with the peak area corresponding to four protons of ethylene group. These spectroscopic data indicated clearly the presence of a succimide group in the structure, *i.e.*, the formation of mono(6-deoxy-6-succimino)-β-CyD (nominal molecular weight 1216, see Chart 3). The release profile of mono(6-deoxy-6-succimino)-β-CyD at pH 7.0 and 25 °C was monitored by TLC. The succimidyl-β-CyD was released at the same rate as that of prednisolone, indicating a single step hydrolysis of prednisolone/β-CyD amide conjugate into prednisolone and the succimidyl-β-CyD.

Figure 3 shows the hydrolysis rate–pH profile of prednisolone 21-hemisuccinate/β-CyD amide conjugate at the pH range of 2—7 and 25 °C. The hydrolysis rate at the acidic region (pH 2—4) was almost the same, but increased with a rise of pH showing a slope of 1.09, indicating an involvement of specific-base catalysis in the hydrolysis. Therefore, the pH-profile was analyzed according to Eq. 1, using a nonlinear least-squares method:

$$k=k_{OH}\cdot(OH^-)+k_0 \quad (1)$$

where k is a first order rate constant for the hydrolysis of prednisolone 21-hemisuccinate/β-CyD amide conjugate, k_{OH} is a second order rate constant for the specific-base catalysis,

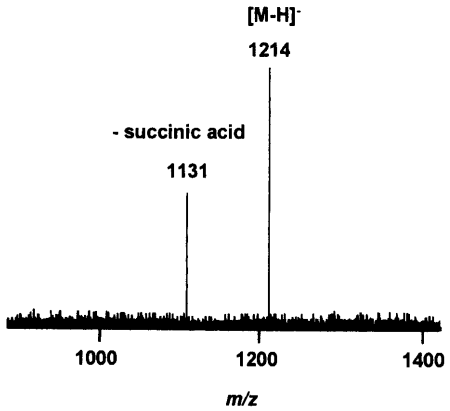

Fig. 2. FAB-Mass Spectrum of Mono(6-deoxy-6-succimino)-β-CyD in Negative Ion Mode

Matrix: DMSO+diethanolamine.

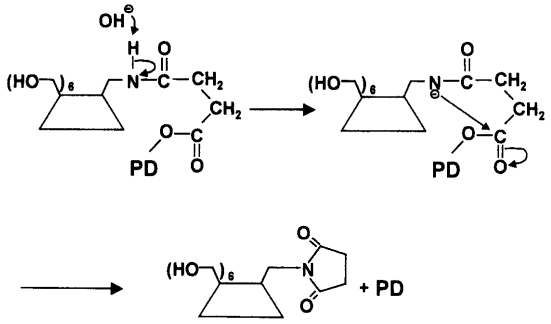

Chart 3. Hydrolysis Mechanism of Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate

PD: prednisolone.

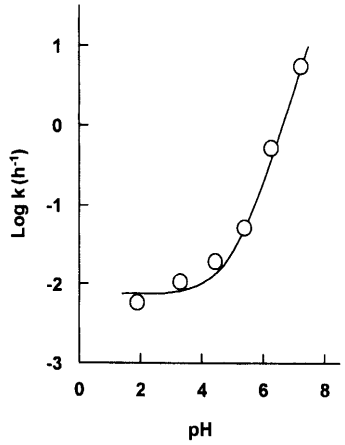

Fig. 3. Hydrolysis Rate–pH Profile of Prednisolone 21-Hemisuccinate/β-CyD Amide Conjugate at pH Range of 2—7 and 25 °C

The solid line is a theoretical curve calculated by a nonlinear least-squares method using Eq. 1.

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k_0 is a first order rate constant for the spontaneous hydrolysis or a second order rate constant for the water catalysis, and (OH^-) is hydroxide ion concentration. The least-squares treatment gave the values of $k_{OH}=4.62\times10^7\,M^{-1}\cdot h^{-1}$ and $k_0=1.16\times10^{-2}\,h^{-1}$, and the experimental data fitted closely to the theoretical curve drawn using these rate constants, as shown in Fig. 3. According to the above results, the hydrolysis mechanism of the β-CyD amide conjugate was postulated as shown in Chart 3. The amide group attacks nucleophilically the terminal ester group, forming a tetrahedral intermediate, in turn releasing prednisolone and the succimidyl-β-CyD simultaneously.

In conclusion, prednisolone 21-hemisuccinate was covalently bound to the amino group of mono(6-deoxy-6-amino)-β-CyD. Prednisolone 21-hemisuccinate/β-CyD amide conjugate was rapidly hydrolyzed to prednisolone and the succimidyl β-CyD with $t_{1/2}$ of 6.50 min in pH 7.0 at 25 °C. The rapid hydrolysis of the conjugate could be ascribed to the involvement of a nucleophilic catalysis of the amide in the reaction. The results suggest that succinic acid, bound to a drug through ester linkage at one carboxylic group and bound to a pro-moiety through amide linkage at another carboxylic group, is useful as a spacer for construction of the immediate release type CyD prodrugs. Because the conjugate readily dissolved in water, it may be useful as an injectable prodrug of prednisolone. Design and evaluation of prednisolone 21-hemisuccinate/β-CyD ester conjugate where both carboxyl groups of succininc acid were bound to the drug and β-CyD through ester linkage are in a research for a slow release type prodrug.

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