Use of 2-Hydroxypropyl-β-cyclodextrin as a Solubilizing and Stabilizing Excipient for Protein Drugs

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A chemically modified, amorphous β-cyclodextrin, namely, 2-hydroxypropyl-β-cyclodextrin (HPCD), was examined as a solubilizing and stabilizing agent for protein drugs. The aqueous solubility of ovine growth hormone at pH 7.4 was increased through the use of HPCD. This effect was manifested by higher UV transparency at 600 nm. Interleukin-2 (IL-2) is rendered insoluble upon lyophilization in the absence of stabilizers. Use of aqueous HPCD provides a clear solution, as indicated by fluorometric light scattering, and inhibits aggregate formation, as shown by ultracentrifugation and Western blot analyses. In addition, there were no major conformational changes of IL-2 in HPCD formulation as indicated by fourth-derivative ultraviolet spectroscopy. Finally, IL-2 retained 100% of its biopotency when prepared in HPCD solutions. Aggregation of insulin was also suppressed by HPCD. These data, as well as the i.v. safety of HPCD and its well-characterized chemical composition, suggest that this starch derivative may be a potentially useful excipient for protein drugs intended for parenteral use.

KEY WORDS: 2-hydroxypropyl-β-cyclodextrin; ovine growth hormone; interleukin-2; insulin; stability.

INTRODUCTION

The explosion in the number of biotechnologically derived protein drugs has presented a variety of novel formulation problems to the pharmaceutical chemist. These problems generally are related to protein instability in aqueous media. Degradation processes such as hydrolytic cleavage, deamidation, racemization, oxidation, and disulfide bond exchange can occur in aqueous protein solutions (1,2). In addition, changes in the three-dimensional protein structure caused by unfolding and "unnatural" refolding, alterations in hydrogen bonding, and changes in hydrophobic interactions may lead to aggregation, precipitation, and loss of biopotency (1,2). The aqueous solubility of the protein can also be limited at physiological pH.

A number of techniques have been examined for stabilizing aqueous protein formulations, especially those intended for parenteral administration, including the use of serum albumin, various amino acids, carbohydrates, surfactants, polyhydroxylated alcohols, and chelating agents (1,2). While these applications have been found to be useful in many circumstances, there are many examples where present technologies have failed to generate adequately soluble or stable preparations with appropriate i.v. safety. One approach to this problem involves the use of chemically modified β-cyclodextrins. These derivatives, unlike the parent β-cyclodextrin, are parenterally safe (3–14). This communication examines the use of 2-hydroxypropyl-β-cyclodextrin (HPCD) (Fig. 1) as a solubilizing and stabilizing excipient for three biologically important peptides: ovine growth hormone (O-GH), interleukin-2 (IL-2), and bovine insulin.

MATERIALS AND METHODS

HPCD was prepared as previously described (8,10). Briefly, β-cyclodextrin was solubilized in aqueous base and treated with propylene oxide. The reaction mixture was then neutralized via ion-exchange chromatography and lyophilized. The crude powder was extracted with acetone to remove traces of propylene glycol and polypropylene glycols which formed during the reaction. The product was collected by filtration and subject to two cycles of hydration/lyophilization. The material was then characterized as to its mean degree of substitution by fast atom bombardment mass spectrometry (FAB-MS). Trace analyses (propylene glycol, unreacted β-cyclodextrin, acetone, halogen, heavy metals, and arsenic) were also completed, as were residue on ignition and specific rotation analyses. The average molecular degree of substitution was calculated to be 7.0. HPCD generated by this process was not pyrogenic as measured by the standard limulus amebocyte lysate (LAL) test. β-Cyclodextrin was obtained from Aldrich Chemical Company. O-GH was obtained from National Institutes of Health, Natural Pituitary Hormone Distribution Program, IL-2 was obtained from Cetus, Inc., Emeryville, CA, and bovine insulin was purchased from Cal Biochem.

Solubilization of O-GH. All solutions of proteins and cyclodextrins were prepared using deionized water (Barnstead Nanopure II Ultrapure Water System, 18.3 Ω). All solutions contained 100,000 IU/liter penicillin, 100 mg/liter streptomycin, and Fungizon (antifungal agent). The pH was adjusted with 0.1 *N* NaOH. UV absorption studies were performed on a Cary 210 spectrophotometer.

Studies with IL-2. Light scattering measurements were made fluorometrically by measuring the amount of light scattered from a sample at 90°C at 510 nm. The instrument (Perkin Elmer) was precalibrated relative to a diluted suspension of 0.07-μm latex beads (Polysciences).

The ultracentrifugation assay was performed using a Beckman L8-70 centrifuge fitted with a type 70.1 Ti rotor. Five milliliters of each formulation was spun at 105,000*g* for 1 hr. IL-2 present in the supernatant was determined by UV absorbance at 280 nm.

Western blot analyses of IL-2 samples were performed as follows: IL-2 samples reduced with β-mercaptoethanol were run on sodium dodecyl sulfate (SDS)–polyacrylamide gels (10%), blotted onto nitrocellulose, and probed with an anti-IL-2 monoclonal antibody (Cetus Corp.), followed by incubation with a goat anti-mouse IgG–horseradish peroxi-

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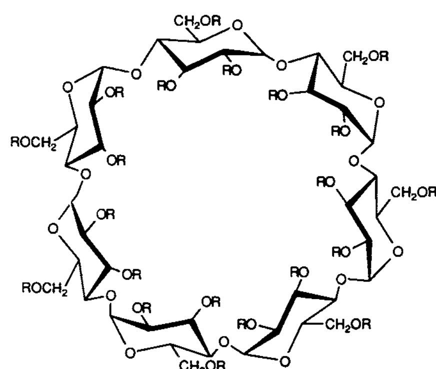
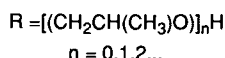

Fig. 1. Schematic representation of isomeric mixtures of 2-hydroxypropyl-β-cyclodextrin.

dase conjugate (Bio-Rad). The nitrocellulose paper was developed using tetramethylbenzidine stain.

Biological activity of IL-2 formulations was measured using an HT-2 cell proliferation bioassay (15). HT-2 cells are derived from an IL-2-dependent murine helper T-cell line and proliferate in a dose-dependent manner with addition of IL-2. Cell proliferation was measured using a spectrophotometric method. Activity of unknown samples was measured relative to a standard included on each assay plate. The standard was calibrated against the World Health Organization IL-2 International reference preparation.

Studies on Bovine Insulin. Insulin solutions (1 mg/ml) were prepared in isotonic pH 7.4 phosphate buffer or buffer containing 40% (w/v) 2-hydroxypropyl-β-cyclodextrin. Samples were stored at room temperature, unprotected from light. One year after preparation of the samples, the fourth-derivative UV spectrum was taken and the buffered test solutions with and without cyclodextrin were compared to a fresh solution of insulin buffer. UV measurements were obtained from a Hewlett-Packard 8451A diode array spectrophotometer. The spectrum was recorded between 250 and 330 nm and a three-point smoothing program was used.

RESULTS AND DISCUSSION

Studies on O-GH

O-GH is a polypeptide containing 188 amino acids with a molecular weight of approximately 20,000 daltons (16). This material is poorly soluble in aqueous media at pH 7.4. This limitation requires that pharmaceutically active concentrations of O-GH be prepared in highly basic media (pH 11) prior to administration. These pH extremes cause pain upon i.v. injection. In addition, the method is not efficient in maintaining complete dissolution. O-GH is poorly soluble in buffer at pH 7.4, as indicated by significant light scattering at 600 nm. In contrast, O-GH at the same concentrations (dissolved in 40%, w/v, HPCD) manifests a significant decrease in light scattering consistent with the formation of clear solutions. When the concentration of HPCD was reduced from 40 to 5% (w/v), the beneficial clarifying effects were maintained, as shown in Table I.

Studies on Interleukin-2

Natural interleukin-2 (IL-2) is a glycoprotein containing 133 amino acids with a molecular weight of 15,420 daltons (17). This lymphokine is produced by T lymphocytes after antigenic stimulation and is necessary for the proliferation and stimulation of activated T cells, natural killer cells, and other components of the immune system (18,19). Recombinant forms of IL-2 have been successfully used in the treatment of various refractory neoplasms (20). This protein is relatively hydrophobic, and as such, turbidity occurs upon lyophilization and reconstitution. Few parenterally safe excipients provide a clear IL-2 solution upon reconstitution.

Interleukin-2 (1 mg/ml) was formulated in 1-ml solutions containing 25 and 12.5% (w/v) HPCD and lyophilized. The freeze-dried preparations were reconstituted with 1 ml of water for injection. On visual observation, the systems were clear. Light scattering, as measured fluorimetrically at 510 nm, was minimal. To determine the minimum level of HPCD needed to yield a clear reconstitute, a range of HPCD concentrations (0–25%, w/v) was included in IL-2 formulations (1 mg) containing 1% sucrose and 10 m*M* citrate buffer and water to yield a total volume of 1 ml. These samples were lyophilized as before and reconstituted with 1 ml water. Light scattering of the samples indicated that concentrations of HPCD between 0.2 and 0.5% (w/v) gave maximal clarity and that higher HPCD levels provided no advantages in this respect. At lower HPCD levels, light scattering increased significantly (Fig. 2). Stoichiometrically, the data suggest that 20–40 molecules of cyclodextrin are interacting with each IL-2 molecule at the inflection point. When these samples were examined by Western blot analysis, no appreciable increase in dimer/aggregate formation was apparent. When unmanipulated IL-2 was centrifuged, the supernatant contained ~93% of the protein. IL-2 formulated with 0.5 or 2% HPCD and 1% sucrose behaved similarly upon reconstitu-

Table I. Effect of HPCD on the Cloudiness of O-GH Solutions as Determined by UV Absorbance at 600 nm ± SD[a]

O-GH (mg/ml)	UV absorbance, HPCD (% w/v) 0	5	10	20	40
1.25	0.219 ± 0.007	0.186 ± 0.003	0.112 ± 0.001	0.068 ± 0.001	0.054 ± 0.001
2.5	0.510 ± 0.009	0.329 ± 0.004	0.260 ± 0.001	0.214 ± 0.003	0.137 ± 0.001
5.0	1.51 ± 0.011	0.512 ± 0.006	0.397 ± 0.004	0.383 ± 0.003	0.438 ± 0.005

[a] Each value represents the mean of three measurements.

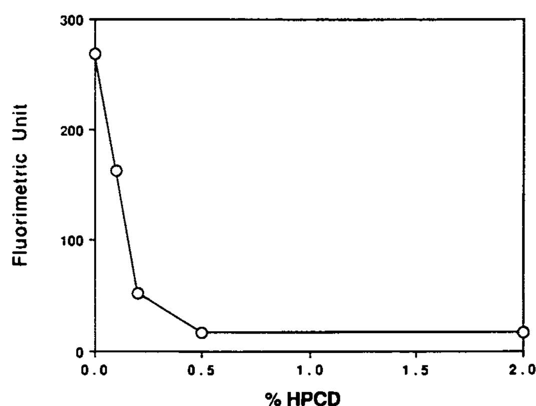

Fig. 2. Effect of various concentrations of 2-hydroxypropyl-β-cyclodextrin on solution clarity of reconstituted IL-2 formulations.

tion, indicating that the protein was present in an unaggregated form.

To assess conformational changes of IL-2 in the presence or absence of HPCD, fourth-derivative ultraviolet spectroscopy (4D-UV) was used (21). This technique can detect environmental changes of component aromatic amino acids and may be useful in demonstrating changes in protein shape (22–25). Figure 3 gives the 4D-UV spectra for IL-2 in two aqueous noncyclodextrin formulations and in a 2% (w/v) HPCD solution. No changes in the spectra occurred, consistent with an unperturbed structure.

The stability of IL-2 formulations was subsequently examined. Solutions of 1 mg/ml IL-2 with varying concentrations of HPCD (0.05–2.0%), sucrose (1–2%), and β-cyclodextrin were prepared and lyophilized. After 1 week of storage at 37°C, these solutions were reconstituted and their clarity was examined by fluorometric light scattering. HPCD-containing formulations and solutions of the unmanipulated protein expressed similar light scattering, indicating that no substantial aggregation occurred. Interestingly, solutions of IL-2 formulated in unmodified β-cyclodextrin were significantly turbid. In addition, Western blot analysis of reducing SDS-PAGE indicated negligible dimer formation in HPCD formulations, while the β-cyclodextrin system exhibited a significant dimer band.

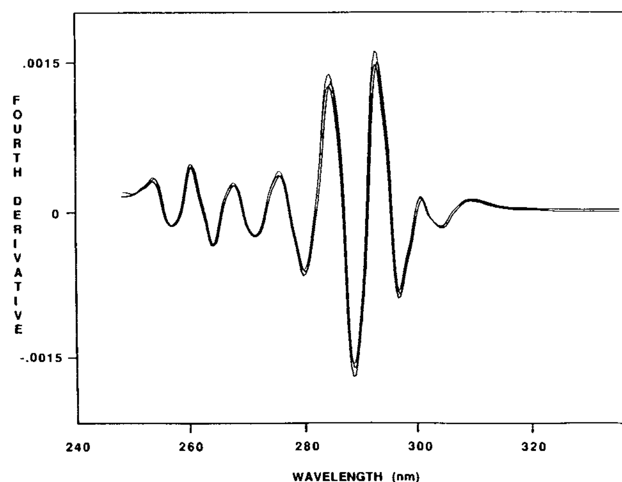

Fig. 3. Fourth-derivative UV spectra of IL-2 in 2-hydroxypropyl-β-cyclodextrin as well as two aqueous formulations.

Table II. Effect of Various Storage Conditions on the Clarity of IL-2 Formulations[a]

Type of formulation	Temp.	Time of storage (days)	Light scattering
Lyophilized powder	4°C	30	66
Lyophilized powder	37°C	30	90
Liquid	25°C	30	26
Liquid	37°C	30	492[b]

[a] Initial formulations contained 1 mg/ml IL-2, 10 m*M* citrate buffer, 2.0% HPCD, 2.0% sucrose and were adjusted to pH 6.5.
[b] Particulate matter appeared.

An IL-2/HPCD formulation (1 mg/ml IL-2, 10 m*M* citrate buffer, pH 6.5, 2% sucrose, and 2% HPCD) was studied in both the lyophilized and the liquid form (Table II). After 30 days of storage of the lyophilized powder at 4 or 37°C, reconstitution yielded clear solutions as indicated by light-scattering techniques. In addition, solutions of the formulation stored at 25°C were clear at the end of 4 weeks. Storage of the liquid at 37°C, however, produced cloudy, aggregated systems. Longer-term studies indicated that no significant dimer formation occurred in the lyophilized product when stored for 12 weeks at 4°C. These samples were analyzed by Western blot methods and visualized using a double antibody technique.

Finally, the effect of HPCD on IL-2 bioactivity was examined using an HT-2 cell proliferation assay (MTT stain), as previously reported (15). Formulations of IL-2 containing 0.05–25% HPCD were lyophilized and reconstituted and examined for their potency. No inhibition of activity was apparent (Fig. 4). Similar results have recently been reported for human growth hormone (h-GH). HPCD acts to stabilize h-GH to agitation induced aggregation and does not mitigate the biological potency of the hormone (26).

Studies on Bovine Insulin

Insulin is a molecular weight 6000 protein containing 51 amino acids and is widely used as an antidiabetic drug. Novel technologies for treating diabetes have included the

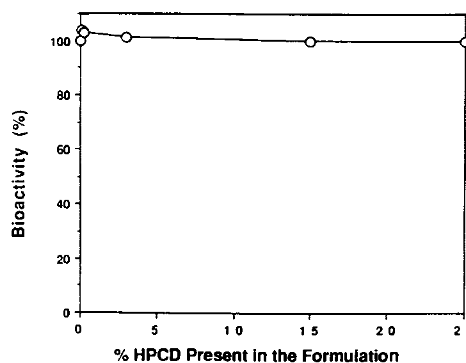

Fig. 4. Bioactivity of IL-2 formulations in various concentrations of 2-hydroxypropyl-β-cyclodextrin. An HT-2 cell proliferation assay was used in this determination.

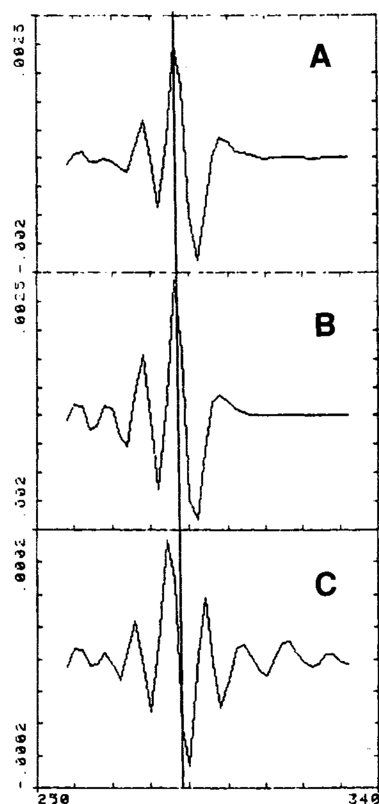

Fig. 5. Fourth-derivative UV spectra of insulin (1 mg/ml) dissolved in either pH 7.4 phosphate buffer (C) or buffer containing 40% (w/v) HPCD (A) after 1 year of storage at room temperature and freshly prepared insulin in pH 7.4 phosphate buffer (B).

development of insulin pumps which deliver a metered portion of insulin parenterally as a function of blood glucose concentrations. Unfortunately, insulin can aggregate in these devices, causing them to clog with the precipitated protein (1). HPCD was examined as a stabilizing excipient. Bovine insulin dissolves in both pH 7.4 buffer and buffer containing 40% HPCD. Within 2 weeks at ambient temperature, unprotected from light, the 1-mg/ml solution of insulin in buffer had begun to precipitate. By 4 weeks the buffered medium was milky, while the HPCD-containing solution remained clear. These samples were stored for 1 year at room temperature. The 4D-UV spectra for a freshly prepared insulin solution and the 1-year-old samples of insulin in HPCD and buffer demonstrated two important differences: the amplitude of the spectra of insulin for 1 year in buffer was considerably lower than that for the two other preparations, and the derivative maxima were shifted in the 1-year/buffered insulin sample relative to the freshly prepared standard or insulin stored in HPCD (Fig. 5). These changes are consistent with significant precipitation in the buffered insulin system, as well as structural deformations relative to the HPCD and freshly prepared materials.

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