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Synthesis, characterization and release of cromoglycate from dextran conjugates

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Summary

This paper describes the synthesis of a macromolecular derivative of the anti-asthmatic drug sodium cromoglycate. Cromoglycic acid (CGA) was covalently bound to dextran (Mol. Wt 10000) by two methods. The first, involving chlorination of the free acid, followed by reaction with dextran in formamide, resulted in low yields (1.5%) of an ester complex (CGA-DEX) containing 2.5% w/w CGA. During hydrolysis experiments in phosphate buffer pH 7.4, this conjugate was found to release cromoglycate with a half-life of 10 h. In the second method, CGA was reacted with dextran via an imidazolide intermediate. This procedure gave higher yields (30–50%) of CGA-DEX, with the complexes containing between 0.8 and 40% w/w CGA, dependent upon reaction conditions. CGA-DEX containing 0.8% w/w CGA released cromoglycate with a half-life of 39 min, whilst another batch containing approx. 40% w/w CGA had a release half-life of 290 min, in buffer of pH 7.4 at 37°C. In contrast to previously reported conjugates of metronidazole and benzoic acid, the dextran conjugates of CGA studied released the drug more rapidly.

Introduction

The ongoing intensive search for novel and innovative drug delivery systems is a consequence of some conventional dosage forms not being sufficiently effective in conveying the drug compound to its site of action and, once in the target area, in releasing the active agent over a desired period of time. Many drugs with good pharmacological properties are limited in usefulness by poor chemical stability, metabolism or lack of organ tropism. Owing to their short half-life, the therapeutic use of some drugs, sodium cromoglycate (SCG) for example, is limited by the need to give frequent doses to the patient. This is often inconvenient and may lead to poor compliance.

SCG is used primarily in the prophylactic treatment of bronchial asthma. Ester derivatives of SCG have previously been synthesized, to increase the lipophilicity of the molecule, these have been tested as prodrugs for dermal delivery with a view to treating allergic skin conditions (Cairns et al., 1972; Bodor et al., 1980).

The ability of dextrans to form a variety of complexes with drugs is closely related to their basic chemical characteristics, such as the high stability of the glucosidic bond and the presence of numerous reactive hydroxyl groups. Drug

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molecules containing carboxylic acid functionalities, such as CGA, may be attached directly to the matrix by a simple esterification process. The hydroxyl groups of dextran can react directly with chlorides of organic acids in the presence of bases such as alkaline hydroxides or pyridine (Molteni, 1985). Direct esterification of carboxylic acid derivatives to dextran may also be achieved using carbodiimides, azides or mixed anhydrides, such as chloroacetic or trifluoroacetic anhydride in a condensation reaction (Jones et al., 1973). The quantity of the bound drug depends upon the drug: polysaccharide ratio in the reaction, chemical reactivity of the drug and degree of activation of the dextran.

Dextran conjugates of various organic acids including acetylsalicylic, nicotinic, and benzoic acids have been described by Papini et al. (1969a,b), in a French patent (1971) and by Larsen and Johansen (1985). A condensation reaction occurs between the chloride of the acid and the polysaccharide in the presence of chloride ion acceptors, such as pyridine or dimethylformamide, in various media including formamide, dimethylsulphoxide and water. Other methods for coupling drugs to polysaccharides include oxidation of the polysaccharide by sodium periodate to form polyaldehyde-dextran, preparation of an azide, and activation of dextran with cyanogen halides or succinic anhydride.

Prodrug derivatives, in which the drug is linked directly to the polymer chain, act as depots releasing the active agent in a predictable manner. In most cases, the rates of regeneration of the parent drug are exclusively governed by pH-dependent hydrolysis. The bulky nature of the matrix renders susceptible linkages within a conjugate inaccessible to enzymatic attack (Johansen and Larsen, 1985; Larsen and Johansen, 1985). This is an important factor to consider when such a complex is introduced into a biological environment.

Little attention has been focused on the physicochemical properties of the conjugates, which are influenced by the synthetic method, although they might affect the in vivo behaviour and the biological activities of the conjugates (Larsen and Johansen, 1989). We have investigated whether the method of conjugate synthesis and degree of substitution affect the release of cromoglycate under in vitro hydrolytic conditions. Two methods were adopted to esterify sodium cromoglycate with dextran (T-10). The first procedure involved formation of the bis-acid chloride of CGA whilst the second involved reaction of CGA with dextran via an imidazolide intermediate.

Materials and Methods

The materials used were of AnalaR grade, except where otherwise specified, and water was glass-distilled. *N′*,*N*-Dimethylformamide (99%), 4-dimethylaminopyridine (99%) and 1-carbonyldiimidazole were obtained from Aldrich, U.K. Dextran (T-10) (av. Mol. Wt 9400; lots 14f-0023 and 53f-0240) and fluorescein isothiocyanate dextran (av. Mol. Wt 10 000) were obtained from Sigma, whilst triethylamine and formamide were of general purpose grade from BDH Ltd, Poole. Sephadex G-25, fine grade for gel filtration, was supplied by Pharmacia whilst disodium cromoglycate B.P. (lot 956862105 s) was a gift from Fisons plc. The phosphate buffer, pH 7.4, used for hydrolysis of CGA-DEX was of the following composition and was always freshly prepared using sodium acid phosphate (0.067 M) and sodium phosphate (0.067 M).

Preparation of cromoglycic acid chloride

Cromoglycic acid (CGA) was isolated after a saturated solution of SCG (5.0 mmol) had been neutralized by addition of glacial acetic acid. The resulting precipitate was filtered, the residue collected, then dried overnight in an oven at 80°C and stored in a desiccator before use.

A mixture of CGA (2.1 mmol) and thionyl chloride (140 mmol) was heated under reflux for 1.5 h. After the mixture had been allowed to stand and cool, dry chloroform (15 ml) and dimethylformamide (5.0 mmol) were slowly added. The solution was then refluxed for a further 1.5 h and left overnight to cool. Excess solvent was removed by rotary evaporation. The

product was washed twice with dry chloroform, then dried by rotary evaporation.

Preparation of dextran cromoglycate conjugate (CGA-DEX)

Method 1 (via acid chloride) Dextran T-10 (0.2 mmol) was dried in an oven at 80°C for 1 h before being dissolved in formamide (20 ml) at 40°C. The mixture was stirred until the dextran dissolved. The cromoglycic acid chloride was added in two portions to the dextran; an orange solution was produced which darkened on exposure to light.

Method 2 (via imidazolide intermediate) A solution containing dextran (0.05 mmol), 4-dimethylaminopyridine (0.16 mmol) and triethylamine (3.6 mmol) in 12 ml of *N'*,*N*-dimethylformamide was prepared and allowed to react for 5 h at room temperature. A second solution, containing CGA (1.5 mmol) in 15 ml of *N'*,*N*-dimethylformamide, was prepared and allowed to react at room temperature for 30 min before addition of 1,1-carbonyldiimidazole (1.42 mmol). This solution was allowed to react for a further 30 min before slowly adding the dextran solution. The final reaction mixture was stirred for 48 h at room temperature, 30 ml of dry chloroform was added and the white precipitate which formed was filtered under vacuum and collected.

The crude reaction mixtures containing CGA-DEX (in 3.0 ml of water), prepared via methods 1 and 2, were purified by gel filtration using a Sephadex G-25 column which was eluted with water. Fractions of 100 drops were collected and samples from tubes 15–30 were combined and freeze-dried overnight in 100 ml round-bottom Quickfit flasks (Edwards freeze dryer, Modulyo). The resulting white solid was stored under vacuum in a desiccator.

Detection of cromoglycate and dextran in conjugates

A 3.0 ml sample of the conjugate, formed by either methods 1 or 2, was loaded onto the gel column, then eluted with water. 100 fractions, each of 5.0 ml, were collected and the presence of cromoglycate in each tube was measured by fluorimetry (Perkin Elmer LS-5 luminescence spectrometer) using excitation and emission wavelengths of 359 and 476 nm, respectively. Excitation and emission slit widths were set at 2.5 and 5.0 nm, respectively.

For detection of dextran, a 1.0 ml sample was pipetted into a test tube and 0.05 ml of phenol 80% w/w added. 5.0 ml of concentrated sulphuric acid was then added rapidly, the stream of acid being directed against the liquid surface to ensure good mixing. The tubes were then left to stand for 10 min before shaking in a water bath at 30°C for 20 min. The absorbance of the solutions was measured at 480 nm (LKB Biochrom Ultrospec 4050, ultraviolet spectrophotometer). The presence of SCG in test solutions did not interfere with the assay.

Hydrolysis of conjugates in pH 7.4 buffer and HPLC assay for cromoglycate

25 mg of dextran conjugate prepared by method 1 was dissolved in 5.0 ml of phosphate buffer pH 7.4. For dextran conjugates of cromoglycic acid prepared by method 2, 10 mg of each conjugate was dissolved in 3.0 ml of phosphate buffer, pH 7.4. The solutions were incubated at 37°C in a water bath for 24 h. At specified time intervals, 200-μl aliquots of solution were removed and assayed for cromoglycate as described below.

The amount of cromoglycate released from the conjugate under different conditions was quantified by HPLC. A C-18 reverse phase column (Spherisorb S5-ODS-2, 250 mm × 4.6 mm) was eluted at 1.0 ml/min with 0.067 M phosphate buffer, pH 7.4, containing 0.064% tetrabutylammonium hydroxide and 40% methanol. Samples were introduced into the column via a Rheodyne 7125 injector with a 100 μl loop and determined by UV absorbance at 238 nm. Peak areas were quantified using a computing integrator (Spectra Physics SP 4290) and amounts of cromoglycate were evaluated by comparison with calibration curves determined on each day of assay. CGA-DEX was eluted with the solvent front whilst cromoglycate had a retention time of 12 min.

Infinity values were determined after hydrolysis for 24 h and did not increase when the conjugate samples were hydrolysed with acid. Hydroly-

sis rate constants were calculated using the infinity values to determine the percentage conjugated at various times.

Results and Discussion

Sodium cromoglycate was neutralized in the presence of strong acid, resulting in the precipitation of CGA. The infrared spectrum of the yellow solid showed a strong carbonyl stretch band at 1725 cm^{-1} and a broad band between 3200 and 3500 cm^{-1} associated with a hydroxyl moiety, thus confirming free acid formation.

Method 1 involved the conversion of the free acid to acid chloride in the presence of thionyl chloride and catalytic amounts of dimethylformamide. The red liquid formed was analysed by infrared spectrometry using a sodium chloride cell. The carbonyl stretch band associated with a free acid was appreciably weaker in this spectrum; this, together with the appearance of a strong band at approx. 1780 cm^{-1}, associated with an acyl halide, confirmed cromoglycic acid chloride synthesis.

Dextran was dissolved in formamide before esterification with the synthesised acid chloride. An ester bond was formed between the polysaccharide and acid chloride, as demonstrated by a slight shift in carbonyl stretch from 1725 to 1770 cm^{-1}, associated with esters of unsaturated acids. The successful synthesis of CGA-DEX was confirmed by gel filtration chromatography (Fig. 1). Chromatography of a solution of fluorescein isothiocyanate (FITC)-labelled dextran (Mol. Wt 10 000) gave an indication of the elution fraction where CGA-DEX would be expected to appear; this was in tubes 15–25. A solution of conjugate was then applied to the same column and cromoglycate determined in each tube by fluorimetric analysis. The presence of cromoglycate in tubes 15–35 and the presence of dextran in tubes 15–25 indicated that CGA-DEX had formed. This conjugation reaction produced some CGA-DEX, however, the yield was very low (1.5%) and it contained only about 2.5% w/w cromoglycate. Following initial hydrolysis experiments in buffer

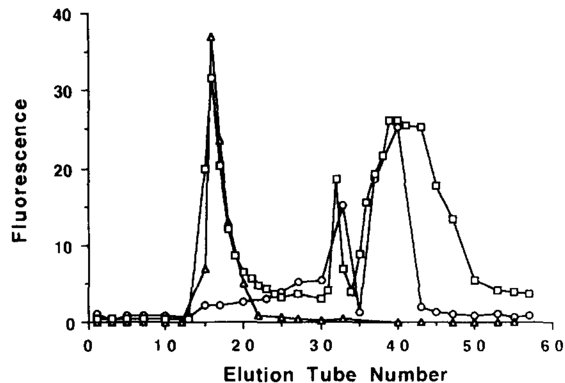

Fig. 1. Chromatographic separation of CGA-DEX (method 1), SCG and FITC dextran. (○——○) SCG in formamide; (△——△) FITC dextran; (□——□) CGA-DEX (method 1).

pH 7.4, the conjugate was found to release cromoglycate, with a half-life of approx. 10 h.

It is well documented that the hydroxyl groups of dextran can react directly with the chlorides of organic acids, in the presence of chloride ion acceptors such as alkaline hydroxides and pyridine, to form esters (Papini et al., 1969a; Molteni, 1979, 1985). Furthermore, numerous lipophilic ester derivatives of CGA (Bodor et al., 1980) and, an immunogen for sodium cromoglycate (Brown et al., 1983) have been formed by utilizing the ability of the free acid to form the bis-acid chloride under appropriate conditions. However, our results show that this method results in only poor yields of dextran conjugate when using CGA.

Method 2, utilizing an imidazolide intermediate, was a better procedure for producing macromolecular derivatives of CGA. The results of gel chromatographic separations indicated that the formation of such complexes was successful. A sample solution of the CGA-DEX prepared by method 2 was applied to the gel column (Fig. 2). The calorimetric test for polysaccharides was applied to samples taken from each eluted fraction. A positive test was given for dextran from tubes 15–35. Fluorimetric analysis of further samples taken from tubes 15–35 indicated that cromoglycate was also present. The covalent linkage between the polysaccharide and CGA was an ester bond, as confirmed by the appearance of a band at approx. 1770 cm^{-1} on the infrared spectrum.

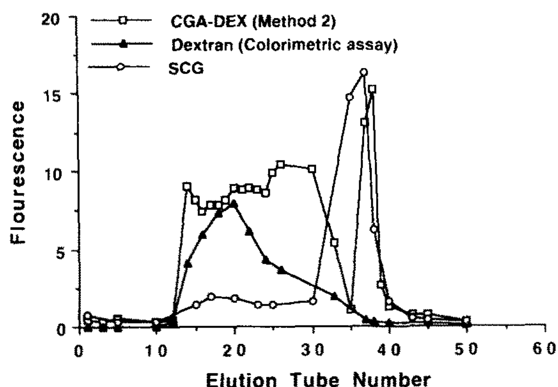

Fig. 2. Chromatographic separation of CGA-DEX (method 2), dextran and SCG. (□——□) CGA-DEX (method 2); (▲——▲) Dextran (colorimetric assay); (○——○) SCG.

Experimental conditions were varied in method 2 to produce CGA-DEX complexes with differing degrees of substitution ranging from 0.8 to 40%. Excellent yields of conjugates were obtained using this second method (30–50%) and initial hydrolysis studies in buffer pH 7.4 showed more favourable release characteristics compared with CGA-DEX prepared by the acid chloride method.

Previous reports indicate that carboxylic acid functions can be introduced into polysaccharide molecules such as dextran by reaction with succinic anhydride (Ferruti et al., 1979) under appropriate conditions. These succinoylation reactions, which are frequently carried out in pyridine, require long reaction times and elevated temperatures. The resulting monosuccinate can then be transformed into a reactive derivative by treatment with 1,1′-carbonyldiimidazole and then subsequently coupled with drugs containing amine or alcohol groups.

It has been demonstrated that dimethylaminopyridine is an excellent agent to promote acylation reactions (Vermeersch and Schacht, 1985) particularly when used in dimethylformamide at 40°C. Carbonyldiimidazole can be used to activate carboxylic acid groups, which under the given reaction conditions, is complete within 20 min. Such a system was previously used to link procainamide and inulin by a succinic acid bridge (Vermeersch et al., 1985).

When metronidazole monosuccinate was esterified with dextran, better reaction with the polyalcohol was achieved using 1,1′-carbonyldiimidazole as a coupling agent with triethylamine and 4-dimethylaminopyridine as coupling promoters (Vermeersch et al., 1985). Cromoglycic acid contains two potentially reactive carboxylic acid groups which may be esterified to dextran under appropriate conditions. The specific solvents and reagents generally used in a typical succinoylation step were employed for the conjugation reaction of cromoglycic acid with dextran (T-10). It is believed that the excess triethylamine can activate the polysaccharide hydroxyl groups through general base catalysis whereas 4-dimethylaminopyridine is known to promote acylation via nucleophilic catalysis.

When concentrations of cromoglycate released hydrolytically from CGA-DEX (prepared by either method) were determined by HPLC analysis the conjugate eluted with the solvent front and had a retention time of about 2 min whereas cromoglycate gave a distinct, reproducible peak at 12 min. At the start of each hydrolysis experiment no free cromoglycate was detectable.

The release of cromoglycate from CGA-DEX in buffer pH 7.4 appeared to be a first-order process. Additionally, the rate of generation of free cromoglycate was dependent upon the degree of substitution (Fig. 3). CGA-DEX prepared by method 1 contained 2.5% w/w cromoglycate which was cleaved from the conjugate with a half-life of approx. 10 h. Batches of conjugate prepared by method 2 contained varying amounts of CGA (0.8–40% w/w), which was released with half-lives ranging from 39 to 290 min. These values were directly related to the degree of CGA substitution (Table 1). CGA-DEX containing 0.8% w/w CGA released cromoglycate with a half-life to 39 min whilst another batch containing approx. 40% w/w CGA had a release half-life of 290 min in buffer pH 7.4 at 37°C. Subsequent batches of CGA-DEX substituted with intermediate amounts of CGA (5.21, 17.60 and 30.83% w/w) had release half-lives of 111, 153 and 218 min, respectively.

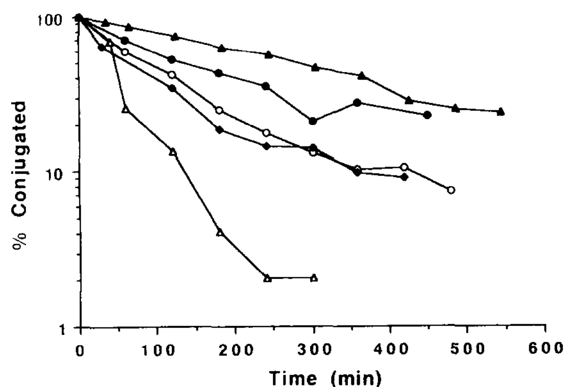

Fig. 3. Release of cromoglycate from CGA-DEX of varying degrees of substitution (DS). DS: (▲——▲) 39.27%; (●——●) 30.83%; (○——○) 17.60%; (◆——◆) 5.21%; (△——△) 0.79%.

The basic knowledge concerning the stability of various dextran ester derivatives originates from kinetic studies performed on model dextran-benzoic acid conjugates. The release data generated were then compared with those obtained from the corresponding aliphatic benzoates (Johansen and Larsen, 1985; Larsen and Johansen, 1985; Larsen et al., 1986). Benzoate esters of dextran (T-70) with varying degrees of substitution (3.4, 9.1 and 15.8% w/w) have been previously synthesized (Larsen and Johansen, 1985) and their rates of hydrolysis at 37°C in pH 7.4 buffer determined. In contrast to our studies with CGA-DEX, these release half-lives of 186, 180 and 182 h, respectively, were found to be independent of degree of substitution. The lability of the CGA-DEX ester bond compared to the dextran benzoic acid ester bond is probably related to the chemical structure of the free acid. Cromoglycate is a strong acid (pK_a 2) which induces electron shifts in the region of the ester bond rendering it more sensitive to hydrolytic cleavage in aqueous solution. The dextran benzoic acid ester bond was found to be more sensitive to hydrolytic cleavage in neutral and alkaline solution than its aliphatic derivatives which was attributed to intramolecular catalysis affected by the neighbouring carbohydrate hydroxyl groups (Larsen and Johansen, 1985).

TABLE 1

Summary of release data for different CGA-DEX batches in phosphate buffer, pH 7.4 at 37°C

Batch of CGA-DEX	Half-life (min)	Degree of substitution (%)
A	39	0.79
B	35	1.62
C	111	5.21
D	137	14.68
E	153	17.60
F	157	22.21
G	218	30.83
H	290	39.27

In vitro release studies have been reported for dextran derivatives of metronidazole and a series of non-steroidal anti-inflammatory drugs in aqueous buffers over a range of pH values. The hydrolysis of these conjugates was much slower than for CGA-DEX. The hydrolysis of dextran-metronidazole monoesters (Mol. Wt 70 000; pH 1.1–9.18 at 37°C) followed first-order kinetics and was susceptible to both specific acid and base catalysis. The release half-lives of metronidazole from such polymeric complexes derived from maleic, succinic and glutaric acids were 1.5, 32.1 and 50.6 h, respectively (Larsen and Johansen, 1987; Larsen et al., 1988). For dextran-naproxen esters synthesized from polymers of Mol. Wt 10 000, 70 000 and 500 000 having degrees of substitution of 9.9, 5.6 and 6.6, respectively, the release half-lives were 179, 175 and 183 h (Larsen and Johansen, 1989). At constant pH and temperature the reactions displayed strict first-order kinetics. The dextrans used to synthesize these derivatives were of different molecular weights (T-10, T-70 and T-500) and contained varying amounts of free drug. Although the release of metronidazole and naproxen from these complexes was characterized in detail with respect to temperature and pH, no data were reported concerning the degree of substitution of a particular drug within a conjugate formed with dextran of a single molecular weight.

In summary, methods have been described for the synthesis of CGA-DEX, conjugates utilizing a dextran of molecular weight 10 000. The conjugates contained between 0.8 and 40% w/w CGA, with degree of substitution being determined by

the reaction conditions. Cromoglycate was released from the conjugates with half-lives ranging from 39 to 290 min, in buffer pH 7.4 at 37°C. Under these conditions the release was dependent upon the amount of the drug contained in the conjugate.

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