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Release of cromoglycate from dextran and inulin conjugates

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Summary

Conjugates (CGA-dex and CGA-inu) were synthesized by esterification of cromoglycic acid to dextran (Mol. Wt 10 000) or inulin (Mol. Wt 5000) via imidazolide intermediates. The resulting conjugates contained 15 and 10% drug, respectively. Under in vitro test conditions the release kinetics of cromoglycate from each conjugate were first-order. In buffer pH 7.4 at 37°C, cromoglycate was released from CGA-dex and CGA-inu with a half-life of 136 and 124 min, respectively. CGA was released more rapidly under simulated physiological conditions, namely, plasma and various concentrations of carboxylic ester hydrolase. At the highest enzyme concentrations studied (approx. 400 U/ml) release half-lives were 88 and 43 min, respectively. The stability of CGA-dex and CGA-inu was reduced considerably in fresh human plasma compared to buffer pH 7.4. The resultant half-lives for CGA-dex and CGA-inu were almost identical at approx. 60 min. Overnight incubation of plasma at 37°C, or storage for 1 month at −20°C, before addition of CGA-dex or CGA-inu, considerably reduced but did not totally abolish its hydrolytic activity. Half-lives for CGA-dex and CGA-inu were 124 and 110 min, respectively, in previously incubated plasma and 115 and 104 min, respectively, in previously frozen plasma.

Introduction

In order to optimize the pharmacological properties of existing drug molecules new formulations have been developed based upon either advanced technological delivery systems, or prodrugs formed by chemical modification of the parent compounds. Both macromolecular and simple aliphatic and aromatic derivatives of a variety of drugs have previously been prepared as a means to achieve a longer duration of pharmacological activity, reduced toxicity, cell-selective therapy, or simply to improve the stability of such compounds during storage.

Dextrans and inulin have been used in medicine for many years, dextrans as plasma expanders and inulin in renal clearance determinations. Their excellent physicochemical properties and the numerous carbohydrate hydroxyl groups available for drug fixation have been exploited in their successful use as macromolecular drug carriers. Several methods, including direct esterification, periodate oxidation and cyanogen bromide activation, have been reported for the conjugation of different drugs to both dextrans and inulin. Covalent linkage of drugs directly to the polymer matrix by esterification has been applied to some therapeutic systems. For example, dex-

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tran and starch have been directly esterified with aspirin (Papini et al., 1969), nicotinic acid (Puglisi et al., 1976) and various non-steroidal anti-inflammatory drugs.

The basic knowledge concerning the stability of dextran ester derivatives originates from comparative kinetic studies with aliphatic esters of benzoic acid (Johansen and Larsen, 1985; Larsen and Johansen, 1985; Larsen et al., 1986). Such investigations showed that the dextran-benzoic acid ester bond was more sensitive to hydrolytic cleavage in neutral and alkaline solutions than the aliphatic benzoates. Furthermore the release rate of benzoic acid from the dextran conjugate was found to be independent of degree of substitution and average molecular weight of the complex.

Practically identical stabilities of various esters of dextran, including benzoic acid and non-steroidal anti-inflammatory conjugates, in aqueous buffer, pH 7.4, and human plasma have been observed (Larsen and Johansen, 1989) suggesting that hydrolysis proceeds without enzymatic catalysis. It was therefore concluded that dextran affords a certain degree of steric protection to any drug which might be fixed close to the polymer matrix.

Esterases play a role in the metabolism of a number of compounds used as drugs in humans. Individual variations in the activity of esterases may therefore be an important factor influencing both the pharmacological and toxic effects of these compounds. The range of substrate specificity of these esterase enzymes ensures the rapid hydrolysis of most ester-based compounds (Mentlein and Heymann, 1984). However, with the widespread and differing distribution of the various enzymes within the body it is difficult to predict the fate of a particular compound. The liver of humans and other mammals contains a variety of carboxylesterases (EC 3.1.1.1) of differing substrate and inhibitor specificities.

This paper details the in vitro release of cromoglycate (CGA) from two polymeric conjugates. The synthesis and characterisation of these analogues has previously been reported (Williams and Taylor, 1992). The release profiles of CGA from the aforementioned inulin (CGA-inu) and dextran (CGA-dex) derivatives are described in buffer pH 7.4 at 37°C. Particular attention is paid to CGA release from each conjugate under more physiological conditions. Thus the release of drug was also characterized in the presence of varying concentrations of carboxylic ester hydrolase and in plasma.

Materials and Methods

Unless otherwise stated, materials used in this work were of AnalaR grade obtained from BDH. Water was glass distilled and esterase (carboxylic-ester hydrolase EC 3.1.1.1 type 1 from porcine liver) was obtained from Sigma. Fresh human whole blood (20 ml) was obtained then centrifuged at 3000 rpm for 15 min. The supernatant plasma was separated and used; immediately, after storage at 37°C overnight in a water bath, or after freezing at −20°C for 1 month and thawing. Ultrafiltration cartridges (Ultracent-10, Mol. Wt cut off 10 000) were obtained from Bio-Rad.

Cromoglycate assay

The amount of cromoglycate released from the conjugates 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 (LDC model III constaMetric pump) with 0.066 M phosphate buffer, pH 7.4, containing 0.064% tetrabutylammonium hydroxide and 40% methanol. Samples were introduced to the column via a Rheodyne 7125 injector with a 100 μl loop and determined by UV absorbance at 238 nm (spectroMonitor III variable wavelength detector, LDC Milton Roy). Peak areas were determined using a computing integrator (Spectra-Physics SP4290) and amounts of cromoglycate were quantified by comparison with calibration curves determined on each day of assay.

Hydrolysis of conjugates

For both the inulin and dextran conjugates of cromoglycic acid, 3.0 mg/ml solutions were prepared in phosphate buffer, pH 7.4, and maintained at 37°C in a water bath for 24 h.

In separate experiments various amounts (approx. 60–400 U/ml) of carboxylic ester hydrolase (EC 3.1.1.1, from porcine liver) were added to the test solutions. The release of cromoglycate from the conjugates was characterized at 37°C over a period of 24 h. At selected time intervals 200 μl aliquots of test solution were taken and assayed for free cromoglycate as previously described.

Hydrolysis of conjugates was further studied in freshly collected human plasma, plasma incubated overnight at 37°C and plasma previously frozen and stored for one month at −20°C. Samples containing 5.0 mg/ml conjugate dissolved in human plasma were placed in a water bath at 37°C and hydrolysis was allowed to proceed. At 60-min intervals, 500 μl aliquots of test solution were taken and diluted with 1.0 ml of phosphate buffer, pH 7.4, in the ultrafiltration cells. The cells were centrifuged at 4500 rpm for 30 min, and the filtrates injected directly onto the chromatograph and analysed for free cromoglycate.

Infinity values were determined after 24 h and were not increased when the conjugate samples were hydrolysed with acid. Hydrolysis rate constants were calculated using the infinity values to determine the percent conjugated at various times. Upon complete hydrolysis CGA-inu (Mol. Wt 5000) was found to contain 10% w/w CGA, whilst CGA-dex contained 15% w/w free drug.

Results and Discussion

The amounts of CGA liberated from CGA-dex and CGA-inu at various time points, under the experimental conditions described, were determined by HPLC assay. The conjugates eluted with the solvent front and had a retention time of 2 min, whereas CGA gave a distinct peak at approx. 12 min. Hydrolysis of CGA-dex and CGA-inu followed apparent first-order kinetics in each environment studied. In buffer pH 7.4 at 37°C, the conjugates were moderately stable, resulting in CGA being released with half-lives of 136 and 124 min, respectively (Tables 1 and 2). The presence of carboxylic ester hydrolase increased the rate of CGA release from CGA-dex and CGA-inu. Consequently, at enzyme concentrations of approx. 400 U/ml, release half-lives were up to two thirds shorter than those determined in buffer, 88 and 43 min, respectively (Tables 1 and 2). At the lowest enzyme concentrations adopted, approx. 60 U/ml, little effect upon CGA liberation from CGA-dex and CGA-inu was noted; the resultant half-lives were 151 and 120 min. The stability of CGA-dex and CGA-inu was considerably reduced in fresh human plasma compared to buffer pH 7.4. The resultant half-lives for CGA-dex and CGA-inu were almost identical, approx. 60 min (Tables 1 and 2). Incubation of plasma at 37°C, before addition of CGA-dex or CGA-inu, reduced but did not abolish its hydrolytic activity. CGA was released from each conjugate with a half-life of 124 and 110 min. Storage of the plasma at −20°C and thawing

TABLE 1

Release half-lives of CGA from CGA-dex containing approx. 75% w/w cromoglycate

Hydrolysis medium	Half-life (min)
Buffer pH 7.4	136
Fresh plasma	59
Plasma previously frozen at −20°C	115
Plasma stored at 37°C overnight	124
Enzyme 64 U/ml	151
Enzyme 127 U/ml	155
Enzyme 254 U/ml	140
Enzyme 275 U/ml	94
Enzyme 339 U/ml	86
Enzyme 424 U/ml	88

TABLE 2

Release half-lives of CGA from CGA-inu containing approx. 10% w/w cromoglycate

Hydrolysis medium	Half-life (min)
Buffer pH 7.4	124
Fresh plasma	66
Plasma previously frozen at −20°C	104
Plasma stored at 37°C overnight	110
Enzyme 60 U/ml	120
Enzyme 122 U/ml	116
Enzyme 284 U/ml	106
Enzyme 333 U/ml	75
Enzyme 426 U/ml	43

prior to use reduced its hydrolytic activity by a similar degree. Characterization of CGA-dex and CGA-inu activity in such environments was necessary before proceeding with in vivo studies involving instillation of conjugate solutions into the rabbit lung. Little attention has been focused on the physicochemical properties of dextran conjugates, which are defined by the synthetic method, although they might affect the in vivo behaviour and the biological activities of the conjugates (Larsen, 1989). CGA-dex and CGA-inu were prepared by the same method and contained similar amounts of CGA. The drug is linked to the macromolecular carrier by an ester linkage via an imidazolide intermediate (Williams and Taylor, 1992). 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 was then compared with that obtained from the corresponding aliphatic benzoates (Johansen and Larsen, 1985; Larsen and Johansen, 1985; Larsen et al., 1986). A benzoate ester of dextran (Mol. Wt 70 000) containing 9.1% w/w benzoic acid was stable to hydrolysis in phosphate buffer at 60°C compared with CGA-dex and CGA-inu (Larsen and Johansen, 1985). The hydrolysis proceeded with the same order of magnitude in 80% human plasma giving a release half-life ($t_{1/2}$) of 183 h. The kinetics of degradation of a dextran-metronidazole-monosuccinate ester conjugate (Mol. Wt 70 000) with degree of substitution of 8.43 at 37°C in buffer pH 7.4 has also been studied (Larsen, 1986; Larsen and Johansen, 1987). Almost identical stability of the conjugate was observed in buffer and 80% human plasma ($t_{1/2} = 32$ h). In each instance it was concluded that hydrolysis in plasma proceeds without enzymatic catalysis due to steric hindrance by the dextran backbone, which prevented cleavage of the ester bond by nonspecific esterases. However, it must be noted that no data were provided concerning prior storage of plasma. We have shown that plasma which is stored at 37°C overnight or has been previously frozen at −20°C had little effect upon the release of CGA from its dextran and inulin derivatives (Tables 1 and 2). Additionally, comparison of CGA release from CGA-dex and CGA-inu in buffer ($t_{1/2} = 136$, 124 min), fresh plasma ($t_{1/2} = 59$, 66 min) and higher concentrations of esterase, approx. 400 U/ml ($t_{1/2} = 88$, 43 min) shows that ester bond cleavage of CGA conjugates is affected by enzymes. This might be explained by the lower molecular weight of the polysaccharide used to form the CGA conjugates affording less protection to the labile ester bond. Dextrans with molecular weight greater than 55 000 are not ultrafiltered through the kidneys, but require depolymerization by α-1,6-glucosidases (dextranases), located predominantly in the liver and spleen before glomerular elimination (Ammon, 1963). The location and nature of the side chains in dextran and the type of dextranases present dictates the efficiency of its fragmentation (Rosenfeld, 1958; Rosenfeld and Saenko, 1964; Walker, 1978; Basedow, 1980). Introduction of a spacer arm linked to dextran through a stable ether bond or periodate oxidation leads to the formation of irreversibly modified dextran carriers whose susceptibility to dextranases decreases with increasing ligand loads (Schacht et al., 1987). The esterase used for this work was isolated from pig liver but was not highly purified, consequently trace amounts of dextranase might affect CGA-dex or CGA-inu breakdown. Human plasma contains no dextranases (Rosenfeld and Lukomskaya, 1957; Walker, 1978) but does contain a variety of other hydrolytic enzymes. Carboxylic acid ester hydrolases (EC 3.1.1) are a group of enzymes which attack molecules such as simple esters, fats and phospholipids (Wills, 1985) and are of particular interest so far as CGA-dex and CGA-inu breakdown is concerned. Cholinesterase (EC 3.1.1.7) and lipase (EC 3.1.1.3) are most prominent in human plasma at activities of 3.8 and 0.14 U/ml, respectively (Wills, 1985). Thus the range (60–400 U/ml) of carboxylic ester hydrolase (EC 3.1.1.1) activity used for in vitro degradation studies on CGA-dex and CGA-inu was far in excess of that normally found in human plasma. Nevertheless, CGA release from the aforementioned conjugates is affected by enzymatic cleavage with release half-lives being reduced (136 to 59 min) for CGA-dex and (124 to 66 min) CGA-inu in the presence of fresh human plasma at 37°C.

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