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The Hemolytic Properties of Chemically Modified Cyclodextrins*

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Abstract. The hemolytic properties of natural cyclodextrins, especially of the more common cyclomaltoheptaose entity, severely hamper their potential use as carriers in pharmaceutical applications where parenteral administration is concerned. A systematic investigation on the role of chemical modifications with regard to the hemolytic character was carried out involving C-6 branched neutral, anionic, cationic and amphoteric derivatives. From these data, conclusions have been drawn about the charge and the geometry of the modification: (i) Substitution at primary hydroxyl groups usually decreases the hemolytic character and the geometry of the substituent affects the hemolytic property; (ii) introduction of an amino group, resulting in a positive charge at physiological pH, decreases the hemolytic character; (iii) negative charges are comparatively less effective in reducing the hemolytic character; (iv) zwitterionic groups seem to enhance the hemolytic character of the cyclodextrin molecule.

Key words: Hemolysis, branched 6-glycosyl cyclodextrins, cationic cyclodextrins, anionic cyclodextrins, amphoteric cyclodextrins.

1. Introduction

Considerable attention is presently being paid to the possibilities offered by cyclomaltooligosaccharides (cyclodextrins, CD) for the parenteral administration of hydrophobic drugs, owing to their solubilization properties, which may result in a higher bioavailability. A main impediment, however, is that cyclodextrins are known to cause hemolysis of human erythrocytes in isotonic solution.

Branched glycosyl cyclodextrins are less hemolytic than the parent cyclodextrin and the decrease in the hemolytic effect appears to be related to the length of the oligosaccharide chain [1]. Among synthetic derivatives, lowering of the hemolytic effect has been noticed for hydroxypropylcyclomaltoheptaose, whereas the methyl

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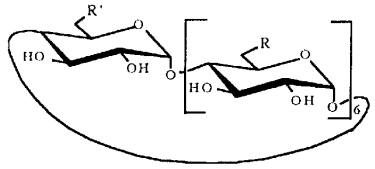

1, R = OH; R' = α-D-Glc-*S*-
2, R = OH; R' = β-D-Glc-*S*-
3, R = OH; R' = α-D-Glc-(1→4)-*O*-α-D-Glc-*S*-
4, R = OH; R' = α-D-Glc-(1→4)-*O*-β-D-Glc-*S*-
5, R = OH;
R' = α-D-Glc-(1→4)-*O*-α-D-Glc-(1→4)-*O*-α-D-Glc-*S*-
6, R = OH; R' = α-D-Glc-(1→4)-*O*-α-D-Glc-*O*-
7, R = OH; R' = NH_2
8, R = R' = CH_2OH-CH_2-NH-
9, R = OH; R' = $CH_2OH(CHOH)_4CH_2NH$-
10, R = OH; R' = $CO_2HCH_2CO_2$-
11, R = OH; R' = CO_2HCH_2NH-

Scheme 1.

ethers are far more hemolytic than the parent CD [2]. The ionic character has furthermore been reported to be of importance with regard to the hemolytic properties since carboxymethylation or succinylation results in low hemolytic products [3]. However, 6^I-amino-6^I-deoxycyclomaltoheptaose was recently reported to be almost as hemolytic as the parent CD [4]. These results point to the importance of structural modifications of the cyclodextrin molecule in order to optimize the solubilization and hemolysis parameters, and the need for further systematic investigation. In the present report, a series of C-6 modified, neutral branched thioglycosides **1–5**, amines **7–9**, carboxylic **10** and amphoteric **11** derivatives (Scheme 1) are assessed comparatively.

2. Experimental

2.1. Materials and methods

Preparative LC was carried out with a Prep LC/250 isocratic pump (Perkin Elmer), equipped with a refractometric LC 30 detector (Perkin Elmer) and a C_{18}-bonded silica column (Nucleosil, 250 × 6.2 mm, 5 μ). Elemental analyses were obtained from samples previously dried at 140 °C/1.33 Pa for 48 h in the presence of P_2O_5. Optical rotations were measured using a Jobin-Yvon Micropolarimeter. FAB Mass spectra (Cs ion gun, acceleration potential 8 kV) were measured in the positive mode with a VG ZAB-SEQ instrument using a 1 : 1 glycerol–thioglycerol matrix. Nuclear magnetic resonance spectroscopy was performed at 500 MHz (^{1}H) or 125 MHz (^{13}C), unless otherwise stated.

2.2. CYCLODEXTRIN DERIVATIVES

6^{I}-*S*-Glycosyl-6^{I}-thiocyclomaltoheptaose derivatives **1–5** [5–8] and 6^{I}-amino-6^{I}-deoxycyclomaltoheptaose **7** [9] were prepared according to literature methods.

2.2.1. *Per (6-deoxy-6-ethanolamino) cyclomaltoheptaose* **8** *[10]*

Per(6-deoxy-6-bromo)cyclomaltoheptaose [11] (8.2 g, 5.2 mmol) was dissolved in 2-aminoethanol (50 mL) and the reaction mixture was stirred for 5 d at room temperature in the dark. It was then poured into acetone (500 mL) and stirred again for 18 h. The resulting precipitate was filtered, washed with acetone and dried. It was then dissolved in water (20 mL) and poured into acetone (250 mL), resulting in a white precipitate which was recovered by filtration, washed with acetone and dried (6.2 g, 87%); TLC (5 : 1 : 5 NH_4OH–BuOH–EtOH), R_f 0.65; FABMS: *m/z* 1436 (100, $[M+H]^+$); ^{1}H-NMR (D_2O): δ5.13 (7 H, H-1), 4.05 (7 H, H-3), 3.99 (7 H, H-5), 3.74–3.74 (14 H, NH—CH_2—CH_2OH, 3.68 (7 H, H-2), 3.55 (7 H, H-4), 3.05–2.92 (14 H, H-6,6′) 2.8 (14 H, NH—CH_2—CH_2OH); ^{13}C-NMR (D_2O): 102.6 (C-1); 83.9 (C-4), 74.0 (C-3), 73.5 (C-2), 71.5 (C-5), 61.1 (NH—CH_2—CH_2OH), 51.7 (NH-CH_2-CH_2OH), 50.3 (C-6). *Anal. Calc.* for $C_{56}H_{105}N_7O_{35}\cdot 2H_2O$: C, 45.68; H, 7.41; N, 6.66. *Found*: C, 45.95; H, 7.25; N, 6.88.

2.2.2. *6^{I}-Deoxy-6^{I}-glucitylaminocyclomaltoheptaose* **9**

D-Glucose (180 mg, 1 mmol) was added to a stirred solution of 6^{I}-amino-6^{I}-deoxycyclomaltoheptaose [9] (**7**, 226 mg, 0.2 mmol) in water (5 mL), followed by $NaBH_3CN$ (100 mg, 1.6 mmol). After 48 h at room temperature the crude product was purified by HPLC (C_{18}, 3: 22 MeOH–H_2O). From the signal ratio (**9**/**9**+**7**), the crude yield was >80%. Collection of the eluates corresponding to **9** and freeze drying resulted in a white foam (180 mg, 69.5%); m.p. 223 °C; $[\alpha]_D$ + 123° (*c* 1.3, H_2O); FABMS (NaI): *m/z* 1299 (100%, $[M+H]^+$), 1321 (52, $[M+Na]^+$); ^{13}C NMR (50 MHz, D_2O): δ 52.19 (C-1 Glc subst.). *Anal. Calc.* for $C_{48}H_{83}NO_{39}\cdot H_2O$: C, 43.80; H, 6.46; N, 1.06. *Found*: C, 43.85; H, 6.43; N, 0.89.

2.2.3. *6^{I}-Deoxy-6^{I}-malonylcyclomaltoheptaose* **10**

Malonic acid sodium salt (340 mg, 2.3 mmol) was added to 6-*O*-*p*-tolylsulfonyl-cyclomaltoheptaose [5] (300 mg, 0.23 mmol) in *N*,*N*-dimethylformamide (5 mL), and the solution was stirred 24 h at 80 °C. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure and poured into acetone (30 mL) resulting in a white precipitate which was recovered by filtration and washed with acetone. It was dissolved in water (5 mL) and layered on the top of a Lewatit MP 580 (OH^-) ion exchange resin column (E. Merck), washed with water (400 mL) and then eluted with 0.1 N HCl (100 mL). Concentration of the acidic eluate at room temperature under reduced pressure and freeze drying of the residue

yielded **10** as a white powder (190 mg, 66%); TLC (5 : 1 : 5 NH_4OH–BuOH–EtOH), R_f 0.65; FABMS: m/z 1243 (100, $[M+ Na]^+$), 1221 (45, $[M+H]^+$). ^{1}H-NMR (95:5 Me_2SO-d_6, D_2O): δ 4.95 (s, 1 H, H-1 subst. Glc residue), 4.8 (s, 6 H, H-1 unsubst. Glc residues), 3.9–3.8 (2 s, 2 H, H-6,6′ ester). *Anal. Calc.* for $C_{45}H_{72}O_{38}\cdot 2H_2O$: C, 42.99; H, 5.89. *Found*: C, 43.15; H, 5.73.

2.2.4. *6^I-Deoxy-6^I-glycinylcyclomaltoheptaose* **11**

N,N-diisopropylethylamine (121 μL, 0.69 mmol) was added to dry glycine ethyl ester hydrochloride (300 mg, 2.33 mmol) in N,N-dimethylformanide (5 mL), and the solution was stirred for 30 min at room temperature. To this solution, 6-O-p-tolylsulfonylcyclomaltoheptaose [5] (250 mg, 0.194 mmol) was then added dropwise with stirring, and the temperature was raised to 70 °C for 18 h with constant stirring in the dark. The reaction mixture was then concentrated under pressure and poured into acetone (100 mL). The resulting precipitate was recovered by filtration and washed with acetone {220 mg (95%); TLC (5 : 1 : 5 NH_4OH–BuOH–EtOH), R_f 0.32; FABMS: m/z 1220 (100, $[M+H]^+$)}. The whole of the recovered material (220 mg, 0.164 mmol) was suspended in water (3 mL) and 2 N LiOH (2 mL) was added. The reaction mixture was stirred for 4 h at 0 °C and then for 18 h at room temperature. It was brought to neutral pH and concentrated under reduced pressure to a solid which was triturated in acetone (200 mL), resulting in a precipitate (150 mg, 77%) which was recovered by filtration, washed with acetone, and dried; FABMS: m/z 1192 (100, $[M+Na]^+$); ^{1}H-NMR (95:5 Me_2SO-d_6 D_2O): δ 7.8 (1 H, NH), 4.9 (1 H, H-1 subst. Glc residue), 4.8-4.7 (6 H, unsubst. Glc residues); ^{13}C-NMR (D_2O): δ 128 (CO_2H), 110 (C- 1 unsubst. Glc), 109 (C-1, subst. Glc), 92 (C-4, subst. Glc), 90 (C-4, unsubst. Glc), 82-69 (C-2, C-3, C-5) 69 (C-6, unsubst. Glc) 67.5 (C-6, subst. Glc), 48 (*C*H_2Gly). *Anal. Calc.* for $C_{44}H_{73}NO_{36}\circ 3H_2O$: C, 42.41; H, 6.35; N, 1.12. *Found*: C, 42.75; H, 6.15; N, 1.38.

2.3. DETERMINATION OF THE HEMOLYTIC ACTIVITY

Human erythrocytes from blood, kept with heparin or EDTA, were separated by centrifugation (1000 × g, 10 min), washed twice with 2 vol. of a normal saline solution and resuspended to give a hematocrit of 20%. Aliquots (0.15 mL) of the erythrocyte suspension were added to the cyclodextrin derivative in 0.9% NaCl solution (final pH 7.4, 0.75 mL) and the suspension was gently agitated for 30 min at 37 °C. It was then centrifuged (1000 × g, 10 min) and the optical density of the supernatant was measured for hemoglobin at 540 nm. Results were expressed as % total hemolysis by comparison with erythrocytes in pure water.

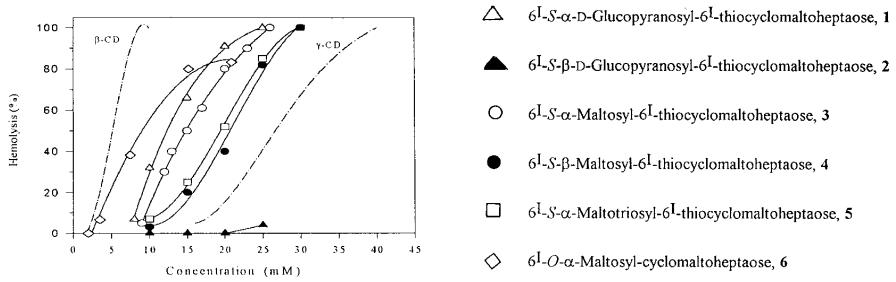

Figure 1. Comparative hemolytic effect for neutral branched 6-*S*-glycosyl-6-thiocyclomaltoheptaoses and the 6-*O*-α-maltosyl analog [1].

3. Results and Discussion

3.1. BRANCHED NEUTRAL 6^I-THIOGLYCOSYL DERIVATIVES

Compounds **1–5**, of interest for the solubilization of hydrophobic drugs [5,7] were found to be less hemolytic than the parent cyclomaltoheptaose, but they were still more hemolytic than cyclomaltooctaose (Figure 1). The hemolytic effect decreases with increasing length of the oligosaccharide chain, i.e., **1** $>$ **3** $>$ **5** in the α series, in agreement with previous results with α-D-glycosyl cyclodextrins [1]. More significant, however, is the fact that β-glycosyl derivatives **2** and **4** are less hemolytic than the α series, and in particular the β-glucosyl derivative **2** is almost non-hemolytic at 20 mmol concentration, suggesting that the geometry of the substituent at C-6 does affect the hemolytic properties. Different hindrance by both derivatives in the approach of the erythrocyte membrane may account for such behaviour.

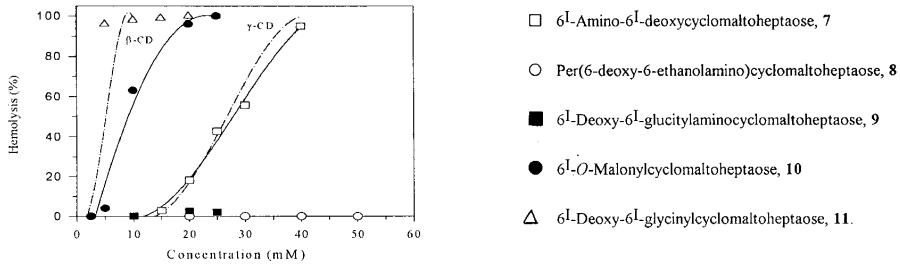

Figure 2. Comparative hemolytic effect with positively charged **7**, **8**, **9**; negatively charged **10**, and amphoteric **11** substituents on cyclomaltoheptaose.

3.2. IONIC DERIVATIVES

Amino-**7**, alkylamino-**8**, **9** and carboxylic **10** cyclomaltoheptaose derivatives were found to be less hemolytic than the parent cyclodextrin (Figure 2). Of special interest are the amine derivatives **7–9** which are even less hemolytic than

cyclomaltooctaose, with almost no noticeable hemolytic effect for per(6-deoxy-6-ethanolamino)cyclomaltoheptaose **8** and 6^I-deoxy-6^I-glucitylaminocyclomaltoheptaose **9**. The result obtained with 6^I-amino-6^I-deoxycyclomaltoheptaose **7**, which is far less hemolytic than cyclomaltoheptaose and in the range of the effect for cyclomaltooctaose, contradicts the report of Leray *et al*. [4], which described **7** as almost as hemolytic as cyclomaltoheptaose. Since the pK_a of the primary **7** and secondary **8**, **9** amines is in the 8–9 range, it is to be expected that the amine functionality in **7–9** is protonated under the conditions used for the hemolysis tests (pH 7.4). A repulsive charge effect with the overall rather amphoteric erythrocyte membrane may then account for the low hemolytic effect. In agreement, the amphoteric glycinyl derivative **11** appears highly hemolytic, even at 3 mmol concentration.

4. Conclusion

Hemolysis of erythrocytes can be ascribed either to induction of an osmotic hypotonic effect or to modification of the membrane composition through extraction of components, cholesterol or phospholipids. The latter hypothesis is more commonly admitted [12] and may involve the interaction of cyclodextrins with a membrane.

Hindrance with C-6 substituents may explain the relatively low hemolytic effect of branched derivatives. Positive charges, which may result from protonation of amines at physiological pH, may enhance the repulsive effect towards the multicharged zwitterionic membrane, an effect which is also seen with anionic derivatives, although to a lesser extent. In agreement with this hypothesis, the zwitterionic derivative **11** is highly hemolytic. Further systematic investigations involving a larger number of representative cyclodextrin derivatives are however needed in order to fully validate this hypothesis.

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