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The Influence of 2-Hydroxypropyl-β-cyclodextrin on the Haemolysis Induced by Bile Acids

R. PANINI, M. A. VANDELLI*, E. LEO*, G. SALVIOLI AND R. CAMERONI*

*Department of Internal Medicine, University of Modena, viale Vittorio Veneto 9, 41100 Modena, and *Department of Pharmaceutical Sciences, University of Modena, via Campi 183, 41100 Modena, Italy*

Abstract

Cyclodextrins improve the water-solubility of drugs and can mask their haemolytic effect in parenteral use. Because the mechanism by which bile acids induce haemolysis is poorly understood, it has been investigated in the presence of 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD).

The haemolytic effect of 1·8 mM solutions of cholic acid, chenodeoxycholic acid (CDCA), deoxycholic acid and ursodeoxycholic acid (UDCA) in isotonic buffer at pH 7·4 was investigated at 37°C in the presence of HP-β-CyD at concentrations from 0·18 to 32 mM. No haemolytic effect was evident for cholic acid and UDCA. The haemolytic effect of the other bile acids was reduced by addition of HP-β-CyD and was prevented at a molar ratio of 1:1 owing to complex formation. An HP-β-CyD:bile acid molar ratio greater than 5:1 had a different effect on the erythrocyte membrane, irrespective of the identity of the bile acid; the effect was in accordance with the complexion affinities.

In the absence of HP-β-CyD, the haemolytic effect of CDCA and deoxycholic acid appeared related to their capacity to form a surface monolayer and to solubilize the components of the erythrocyte membrane. The haemolytic effect observed after complexation of the bile acids appeared to be solely the effect of HP-β-CyD, which was able to form a reversible inclusion complex with lipophilic components of the erythrocyte membranes at concentrations higher than 12 mM.

Cyclodextrins have the capacity to include poorly water soluble compounds in their cavities to form a complex which, because of its hydrophilic exterior, facilitates the dissolution of the guest molecule. This property is widely used in the pharmaceutical field to enhance the water-solubility of drugs.

Derivatives of natural cyclodextrins have been studied both to improve their water solubility and to reduce their toxic effect. Among the proposed derivatives, 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) is one of the most interesting because of its high water solubility and its low toxicity both in-vitro (Leroy-Lechat et al 1994) and in animals (Brewster et al 1990). These properties have suggested the potential parenteral use of this cyclodextrin.

In addition to improving water solubility, complexation with cyclodextrins can mask undesirable drug properties, e.g. the haemolytic effect in parenteral use. The role of cyclodextrins in protecting against the haemolysis produced by drugs or absorption enhancers has so far received little attention (Uekama et al 1981; Jabbal Gill et al 1994).

Few data are available on the mechanism underlying the haemolysis induced by bile acids. One proposed mechanism is the solubilization of erythrocyte membrane constituents at appropriate bile acid concentrations (Lowe & Coleman 1981). The solubilizing effect of bile acids could be related to the surface tension produced. On the basis of these premises, the aim of this work was to investigate the protective effect of HP-β-CyD on the haemolysis induced by different bile acids. The effect on both the haemolysis and the surface tension of different bile acids has therefore been investigated in the presence of HP-β-CyD.

Correspondence: R. Cameroni, Department of Pharmaceutical Sciences, University of Modena, via Campi 183, 41100 Modena, Italy.

Materials and Methods

Materials

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; MW 408·6), chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid; MW 392·6), deoxycholic acid, (3α,12α-dihydroxy-5β-cholan-24-oic acid; MW 392·6) and ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid; MW 392·6) were purchased from Sigma (St Louis, USA). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CyD) (average molar substitution 0·6; average MW 1380) was obtained from Aldrich (Milwaukee, USA). Tris(hydroxymethyl)aminomethane (Tris) (Aldrich) and sodium chloride (Carlo Erba, Milan, Italy) were used to prepare the isotonic buffer. All the materials and chemicals were used as received from the manufacturers.

Haemolysis procedure

Blood from healthy human subjects was drawn into heparinized Vacutainer tubes. Blood samples were centrifuged and the plasma buffy coat was removed. The erythrocytes were then washed three times with 15 mM Tris buffer (pH 7·4) containing 145 mM NaCl and 5 mM glucose; they were then used within 3 h (Salvioli et al 1993).

To evaluate the haemolytic effect, solutions of HP-β-CyD (0·18–32 mM) or bile acids (1·8 mM) in the presence of HP-β-CyD (0–32 mM) were prepared in 50 mM Tris buffer (pH 7·4) containing 145 mM NaCl (isotonic Tris buffer); erythrocytes were incubated in the solutions (final volume 5 mL) at 1% haematocrit at 37 ± 0·5°C for 45 min. The samples were then centrifuged at 5000 rev min^{-1} for 4 min (model 153 Microfuge; Beckmann Instruments, Fullerton, CA, USA). The percentage of haemolysis was evaluated by comparing the absorbance at 546 nm (Lambda 3A; Perkin-Elmer, Norwalk,

USA) of a 100-fold serial dilution of the supernatant with that of erythrocytes totally lysed in distilled water.

The basal value of the haemolysis was estimated by incubating erythrocytes with the pH 7·4 isotonic Tris buffer alone. Both the total and the basal value of haemolysis were determined for each experiment. The haemolysis produced for each sample test was determined in triplicate.

Determination of the mean corpuscular volume

The mean corpuscular volume of the erythrocytes was measured in an S-plus Coulter Counter Analyzer (Coulter, Hialeah, USA). All reported data are averages from three experiments.

Determination of the cholesterol content of the erythrocytes

The cholesterol content of the erythrocytes was evaluated by gas-liquid chromatography using 5α-cholestane (Sigma) as internal standard (Salvioli et al 1985). The amount of cholesterol removed during the incubation was expressed as percent of total cholesterol of red blood cells. All reported data are averages from three experiments.

Surface tension measurements

Surface activities of the pH 7·4 isotonic Tris buffer solutions (25 mL) containing HP-β-CyD (0–32 mM) or bile acid (1·8 mM) in the presence of HP-β-CyD (0–32 mM) were determined at 37 ± 0·5°C. Experiments were performed with a Dognon-Abribat surface tensiometer (Prolabo, Paris, France; accuracy 0·05 dyne cm^{-1}) on the basis of the concept of Wilhelmy. At least 15 measurements were made for each test sample.

Solubility studies

Solubility studies were performed according to Higuchi & Lach (1954). To determine bile acid solubility, excess drug (250 mg) was added to isotonic Tris buffer (5 mL; pH 7·4) containing different concentrations of HP-β-CyD (0–40 mM). The suspensions were shaken in 10-mL screw-capped vials at 30 strokes min^{-1} at 25 ± 1°C. When equilibrium had been reached (after approx. 48 h), the content of each vial was filtered through a cellulose nitrate membrane (pore size 0·45 μm; Sartorius, Göttingen, Germany). The concentrations of the bile acids were determined in the filtered solutions by HPLC as previously reported (Vandelli et al 1995). All reported data are averages from three determinations.

The (1:1) apparent stability constants of the inclusion complexes (k′) were calculated from the straight lines of the phase solubility diagrams of each bile acid in aqueous HP-β-CyD solutions according to Higuchi & Kristiansen (1970):

$$k' = S/[C_S(1 - S)] \quad (1)$$

where C_S (the intercept) is the bile acid solubility in the absence of HP-β-CyD and S is the slope of the straight line.

Results and Discussion

Fig. 1 shows the haemolytic effect of HP-β-CyD on human erythrocytes in the pH 7·4 isotonic Tris buffer. At a concentration of 1·8 mM, HP-β-CyD caused little haemolytic effect (approx. 1·5%). This haemolytic effect is higher than that reported by Jabbal Gill et al (1994), who used rat erythrocytes (0·5% at 4 mM). To explain the difference between the data, it would be remembered that red blood cells from different

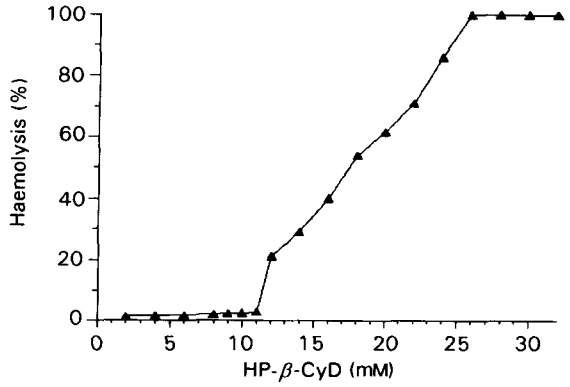

FIG. 1. Haemolysis of red blood cells from healthy human subjects induced at 1% haematocrit at 37 ± 0·5°C by different concentrations of 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) in 50 mM Tris buffer (pH 7·4) containing 145 mM NaCl.

mammalian species have different phospholipid compositions (Nelson 1967; Smith et al 1979; Salvioli et al 1993), mean corpuscular volumes (Smith et al 1979) and osmotic resistance (Coldman et al 1970).

The haemolytic effect of HP-β-CyD becomes evident (10·3%) at a 12 mM and complete haemolysis (100%) is reached at 26 mM. Irie et al (1982) observed cyclodextrin-induced changes in erythrocyte shape in the form of internalization of membranes with release of their components. The cyclodextrin-induced lysis is probably a result of rapid and reversible formation of inclusion complexes in which the lipophilic components of the erythrocyte membrane are partially included into the cyclodextrin cavity (Ohtani et al 1989; Arima et al 1990).

The importance of the inclusion of the lipophilic components in the cyclodextrin cavity is reported in Fig. 2, which shows the amounts of membrane cholesterol (50% in mol total lipid content of erythrocyte membrane) solubilized by increasing concentrations of HP-β-CyD. Very low solubilization of membrane cholesterol occurs at HP-β-CyD concentrations lower than 10 mM. The haemolytic effect appears when cholesterol is solubilized in greater amounts, indicating damage to the membrane.

A haemolytic effect related to the effect on surface tension of the increased HP-β-CyD concentration can, however, be excluded. In fact, as shown by Fig. 3, the dependence of HP-β-CyD-induced surface tension on HP-β-CyD concentration is negligible. The surface tension of the isotonic Tris buffer, 65 dyne cm^{-1}, drops to 55 dyne cm^{-1} at 4 mM HP-β-CyD (2% haemolysis) remaining practically constant until 32 mM (100% haemolysis).

Fig. 4 shows the haemolytic effect of bile acid (1·8 mM) on human erythrocytes in the presence and absence of HP-β-CyD in isotonic Tris buffer. Detergent bile acids induce volume changes in the erythrocytes, which change from discocytes to echinocytes (Salvioli et al 1985); during the incubation the mean corpuscular volume increases from 90 ± 3 to 109 ± 5 μm^3.

Among the bile acids tested, CDCA and deoxycholic acid appear the most potent lytic agents, causing 100% lysis. Practically no lytic effect were observed for cholic acid and UDCA. Because the concentration of bile acid used in this work (1·8 mM) is lower than the critical micellar concentration

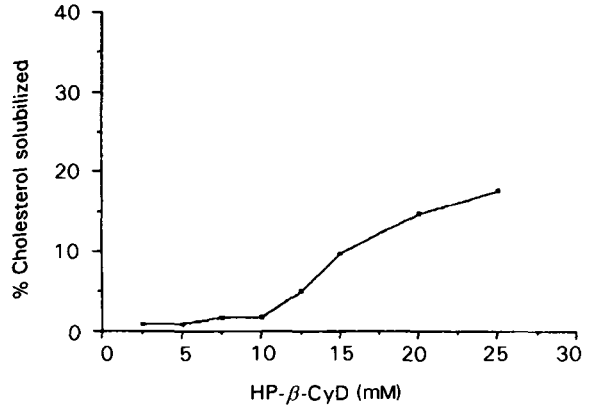

FIG. 2. Solubilization of erythrocyte membrane cholesterol by 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) (mean of three determinations). Conditions as for Fig. 1.

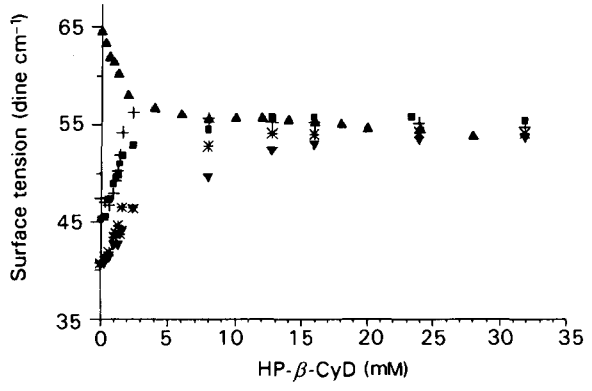

FIG. 3. Surface tension of different concentrations of 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) at 37 ± 0·5°C in 50 mM Tris buffer (pH 7·4) containing 145 mM NaCl in the absence (△) and in the presence of 1·8 mM cholic acid (■), chenodeoxycholic acid (*), deoxycholic acid (▼) and ursodeoxycholic acid (+).

(CMC) (Roda et al 1983), the observed haemolytic effect cannot be a result of extraction of the erythrocyte membrane components into micelles. In fact, at concentrations lower than the CMC, the solubilizing effect of detergents such as bile acids is solely a consequence of their incorporation into cell membranes and then to the solubilization of their components (Lowe & Coleman 1981).

Haemolytic bile acids (CDCA, deoxycholic acid) reduce the surface tension of the pH 7·4 Tris buffer (65 dyne cm^{-1}) to values below 45 dyne cm^{-1} (Fig. 2); surface tension values higher than 45 dyne cm^{-1} are obtained with the non-haemolytic bile acids (cholic acid and UDCA). The different effects on the surface tension could thus explain the haemolytic effect of the bile acid. The greatest reduction of surface tension induced by CDCA and deoxycholic acid suggests the greatest capacity of these bile acids to form a surface monolayer at the erythrocyte membrane and, therefore, the greatest chance of incorporation. CDCA and deoxycholic acid are, in addition, more hydrophobic than cholic acid and UDCA (Roda et al 1990; Panini et al 1994); they are, therefore, more likely to solubilize lipid membrane components. As Fig. 5 shows, deoxycholic acid solubilizes about 70% of total erythrocyte cholesterol.

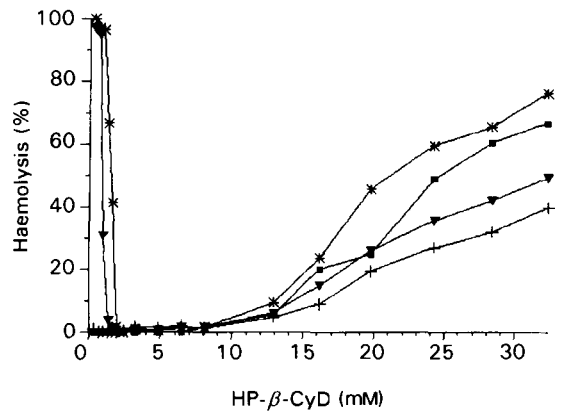

FIG. 4. Haemolysis of red blood cells from healthy human subjects induced at 1% haematocrit at 37 ± 0·5°C by different concentrations of 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) in 50 mM Tris buffer (pH 7·4) containing 145 mM NaCl in the presence of 1·8 mM cholic acid (■), chenodeoxycholic acid (*), deoxycholic acid (▼) and ursodeoxycholic acid (+).

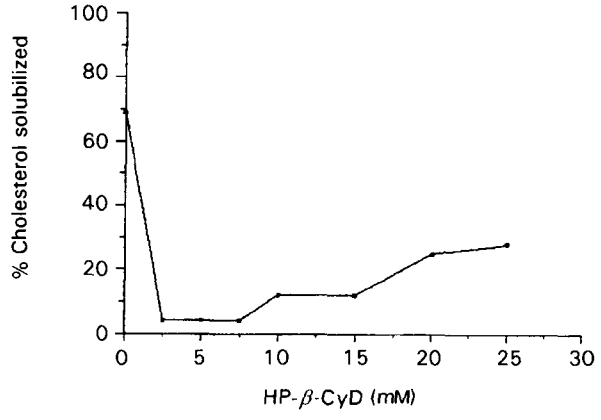

FIG. 5. Solubilization of erythrocyte membrane cholesterol by 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) in the presence of 1·8 mM deoxycholic acid (mean of three determinations). Conditions as for Fig. 1.

The haemolytic effect of both CDCA and deoxycholic acid is significantly reduced by addition of HP-β-CyD until the HP-β-CyD:bile acid molar ratio is 0·8–1·3; at this level the effect is prevented and no change of the mean corpuscular volume is observed. As the molar ratio necessary to effect complete protection of the erythrocytes is about 1:1, the protective effect of HP-β-CyD could be related to complex formation. Bile acids and HP-β-CyD, in fact, form typical 1:1 complexes with different apparent solubility constants (CDCA complex $k' = 990\ M^{-1}$; cholic acid complex $k' = 60\ M^{-1}$; deoxycholic acid complex $k' = 3700\ M^{-1}$; UDCA complex $k' = 700\ M^{-1}$). Saenger & Müller-Fahmow (1988) suggested that cyclodextrins are able to remove amphiphilic molecules from the surface monolayer. Hence complexation of bile acid, preventing the formation of the surface monolayer, should prevent the haemolytic effect of CDCA and deoxycholic acid, because they become unable to insert themselves in the erythrocyte membrane and to solubilize the lipidic components. This hypothesis is confirmed by the observation of the amount of cholesterol solubilized by deoxycholic acid in the presence of

HP-β-CyD. A large reduction of the percentage of cholesterol solubilized was, in fact, observed at a 1:1 molar ratio (Fig. 5).

Cholic acid and UDCA are not haemolytic at the concentration used in this study (1·8 mM) and obviously no haemolytic effect is evident before complex formation because HP-β-CyD does not produce haemolysis at the concentration necessary to form a 1:1 complex.

Increasing the HP-β-CyD concentration results in a different haemolytic effect; this is evident for all the bile acids at an HP-β-CyD:bile acid molar ratio higher than 5:1.

The tendency of cyclodextrin–bile acid complexes to dissociate in aqueous solution, releasing the guest molecule, will depend on the magnitude of their stability constants (Uekama et al 1979). Thus because the species (bile acid, HP-β-CyD and inclusion complex) are in equilibrium in the solution, the haemolytic effect after complex formation (Fig. 1) should be related to the different complexation affinities of the bile acid. Considering the two haemolytic bile acids (CDCA and deoxycholic acid), the haemolytic effect in the presence of HP-β-CyD is higher for CDCA than for deoxycholic acid. As the complex between CDCA and HP-β-CyD has a lower complexation affinity (k′), the greater haemolytic effect of CDCA complex is a result of the greater amounts of free CDCA and HP-β-CyD molecules in equilibrium in the solution. On increasing the HP-β-CyD concentration, the increase in the haemolytic effect should arise mainly as a result of the free HP-β-CyD in the solution, the cavities of which are obviously not occupied by guest molecules (Koizumi et al 1987). In our opinion, in fact, the different solubilizing effect on erythrocyte membrane components, hypothesized for CDCA and deoxycholic acid, bearing in mind the effect on the surface tension, could not be considered, because the same surface tension value is reached for all the bile acids at an HP-β-CyD:bile acid molar ratio higher than 12:1. On the other hand, as shown in Fig. 2, the solubilizing effect of HP-β-CyD on erythrocyte membrane cholesterol is evident for HP-β-CyD concentrations greater than 10 mM.

Considering the two bile acids (UDCA and cholic acid) which are not haemolytic in the absence of HP-β-CyD, the haemolytic effect resulting after the complexation should be solely the effect of HP-β-CyD. In this circumstance the different haemolytic effect can again be related to the different complexation affinities. In fact, the formation of the UDCA–HP-β-CyD complex results in less haemolysis as more HP-β-CyD is engaged in the formation of the complex.

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References

Arima, H., Wakamatsu, K., Aritomi, H., Irie, T., Uekama, K. (1990) Improvement in nasal delivery of insulin with simultaneous use of chemically modified cyclodextrins in rats. In: Duchêne, D. (ed.) Fifth International Symposium on Cyclodextrins. Editions de Santé, Paris, pp 487–490

Brewster, M. E., Estes, K. S., Bodor, N. (1990) An intravenous toxicity study of hydroxypropyl-β-cyclodextrin a useful drug solubilizer, in rats and monkeys. Int. J. Pharm. 59: 231–243

Coldman, M. F., Gent, M., Good, W. (1970) Relations between osmotic fragility and other species-specific variables of mammalian erythrocytes. Comp. Biochem. Physiol. 34: 759–772

Higuchi, T., Lach, L. (1954) Investigation of some complexes formed in solution by caffeine: IV. Interactions between caffeine and sulfathiazole, sulfadiazine, *p*-aminobenzoic acid, benzocaine, phenobarbital, and barbital. J. Am. Pharm. Assoc., Sci. Ed. 43: 349–354

Higuchi, T., Kristiansen, H. (1970) Binding specificity between small organic solutes in aqueous solution: classification of some solutes into two groups according to binding tendencies. J. Pharm. Sci. 59: 1601–1608

Irie, T., Otagiri, M., Uekama, K., Ohtani, Y., Yamada, Y., Sugiyama, Y. (1982) Cyclodextrin-induced haemolysis and shape changes of human erythrocytes in vitro. J. Pharmacobiodyn. 5: 741–744

Jabbal Gill, I., Illum, L., Farraj, N., De Ponti, R.(1994) Cyclodextrins as protection agents against enhancer damage in nasal delivery systems I. Assessment of effect by measurement of erythrocyte haemolysis. Eur. J. Pharm. Sci. 1: 229–236

Koizumi, K., Okada, Y., Kubota, Y., Utamura, T. (1987) Inclusion complexes of poorly-soluble drugs with glucosyl-cyclodextrin. Chem. Pharm. Bull. 35: 3413–3418

Leroy-Lechat, F., Wouessidjewe, D., Andreux, J. P., Puisieux, F., Duchêne, D. (1994) Evaluation of cytotoxicity of cyclodextrins and hydroxypropylated derivatives. Int. J. Pharm. 101: 97–103

Lowe, P. J., Coleman, R. (1981) Membrane fluidity and bile salt damage. Biochim. Biophys. Acta 640: 55–65

Nelson, G. J. (1967) Lipid composition of erythrocytes in various mammalian species. Biochim. Biophys. Acta 144: 221–232

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., Pitha, J. (1989) Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. Eur. J. Biochem. 186: 17–22

Panini, R., Vandelli, M. A., Forni, F., Pradelli, J. M., Salvioli, G. (1994) Complexation with cyclodextrins (CD) as a model for the study of the physicochemical properties of bile acids (BA). J. Hepatol. 21: S92

Roda. A., Hofman, A. F., Mysels, K. J. (1983) The influence of bile salt structure on self association in aqueous solution. J. Biol. Chem. 258: 6362–6370

Roda, A., Minutello, A., Angelotti, M. A., Fini, A. (1990) Bile acid structure-activity relationship: evaluation and bile acid lipophilicity using 1-octanol/water partition coefficient and reversed phase HPLC. J. Lipid Res. 31: 1433–1443

Saenger, W., Müller-Fahmow, A. (1988) Cyclodextrins increase surface tension and critical micelle concentrations of detergent solutions. Angew. Chem. Int. (Engl. edn) 27: 393–394

Salvioli, G., Lugli, R., Pradelli, J. M. (1985) Cholesterol absorption and sterol balance in normal subjects receiving dietary fiber or ursodeoxycholic acid. Dig. Dis. Sci. 30: 301–307

Salvioli, G., Gaetti, E., Panini, R., Lugli, R., Pradelli, J. M. (1993) Different resistance of mammalian red blood cells to haemolysis by bile salts. Lipids 28: 999–1003

Smith, J. E., Mohandas, N., Shohet, S. B. (1979) Variability in erythrocyte deformability among various mammals. Am. J. Physiol. 5: H725–730

Uekama, K., Hirayama, F., Esaki, K., Inoue, M. (1979) Inclusion complexes of cyclodextrins with cinnamic acid derivatives: dissolution and thermal behavior. Chem. Pharm Bull. 27: 76–79

Uekama, K., Irie, T., Sunada, M., Otagiri, M., Iwasaki, K., Okano, Y., Miyatya, T., Kasé, Y. (1981) Effects of cyclodextrins on chlorpromazine-induced haemolysis and central nervous system responses. J. Pharm. Pharmacol. 33: 707–710

Vandelli, M. A., Salvioli, G., Mucci, A., Panini, R., Malmusi, L., Forni, F.(1995) 2-Hydroxypropyl-β-cyclodextrin complexation with ursodeoxycholic acid. Int. J. Pharm. 118: 77–83