Biochemical Pharmacology, Vol. 45, No. 5, pp. 1011–1016, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

PROTECTION OF ERYTHROCYTES AGAINST HEMOLYTIC AGENTS BY CYCLODEXTRIN POLYSULFATE

PAUL B. WEISZ,*†‡ KATHLEEN KUMOR* and EDWARD J. MACARAK†§

Departments of *Chemical Engineering and †Bioengineering, University of Pennsylvania, Philadelphia, PA 19104; and §Connective Tissue Research Institute, Philadelphia, PA 19104, U.S.A.

(*Received* 21 *August* 1992; *accepted* 14 *December* 1992)

Abstract—Cyclodextrins generally exhibit hemolytic activity, some at concentrations as low as 1–10 mg/mL or lower. However, we found previously that a highly polysulfated cyclodextrin has no demonstrable hemolytic activity (Macarak *et al.*, *Biochem Pharmacol* **42**: 1502–1503, 1991). In the present study, we determined that, in fact, cyclodextrin polysulfate (CDS) actively protected erythrocytes against hemolysis induced by a wide spectrum of hemolytically active substances, ranging from pharmaceuticals, such as chlorpromazine, to solid suspensions of siliceous particles. The protective action was also effective against the hemolytic action of non-sulfated cyclodextrins. The similar kinetic responses of the erythrocytes to CDS protection against such chemically and structurally diverse hemolytic agents suggest a common mechanism involving the cell. Addition of the sulfated cyclodextrins to other cyclodextrin compounds used to solubilize poorly soluble pharmaceutical agents can extend the allowable maximum dosage without deleterious hemolytic action.

After sulfating a majority of their hydroxyl groups, cyclodextrin polysulfates (CDS)‖ have been shown to possess potentially important biological activities similar and sometimes superior to those of heparin: (a) anti-angiogenic activity in combination with suitable "angiostatic" steroids [1, 2]; (b) strong binding affinity for fibroblast growth factor [3]; (c) a capability of inhibiting cellular invasion by HIV retrovirus [4, 5]; (d) promotion of endothelial cell and inhibition of smooth muscle cell proliferation [5]; (e) metachromatic activity similar to that of heparin [5]; and (f) a similar ability to complex with protamine [5]. Contrary to the case of other (non-sulfated) cyclodextrins, we found CDS to have no measurable hemolytic activity [6]. We now report that, beyond having no hemolytic activity itself, CDS protects erythrocytes from the actions of a large variety of hemolytic agents.

Cyclodextrins are cyclic oligosaccharides of 6, 7, or 8 glucopyranose units (α, β, or γ-cyclodextrins, respectively) [7, 8]. Their hydrophobic cavities provide opportunities for unique complexing with other molecules, while their peripheral structure containing a large number of hydroxyl groups provides water solubility. This combination of structural features permits cyclodextrins to encapsulate and thereby "solubilize" pharmaceutically active agents of otherwise limited solubilities [7–10]. Parent cyclodextrins and various substituted derivatives, especially the *O*-methyl-substituted CD, called DIMEB [8–10], and *O*-2-hydroxypropyl- [11] and *O*-2-hydroxyethyl-substituted [12] cyclodextrins, have been described for the complexing and solubilization of pharmacological agents. Such complexing can also lower the hemolytic activity of an agent by reducing its (i.e. uncomplexed) concentration in the solution. Such action has been demonstrated for chlorpromazine and other thiazines [13–16], flufenamic acid [15], flurbiprofen [17], and protriptyline [18].

There is, however, a limit to the amount of hemolytic agent that can be solubilized by cyclodextrins. For such applications the cyclodextrins must be used at at least equal molar concentration; however, they will themselves cause hemolysis [19–22], some at concentrations as low as 1–10 mg/mL.

In this report, we demonstrate a general protective capability of *polysulfated* cyclodextrin against hemolytic attack on erythrocytes by a variety of agents. This protective effect extends also to limiting the hemolytic action of (non-sulfated) cyclodextrins.

MATERIALS AND METHODS

β-Cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (β-CD-4Pr) as well as β-cyclodextrin-tetradecasulfate sodium (β-CD-14S) were supplied by G. Reed, American Maize Products, Hammond, IN; heptakis-(2,6-*O*-methyl)-β-cyclodextrin (β-CD-14M), generally known as DIMEB, was supplied by J. Sejtli of Chinon Pharmaceutical and Chemical Works, Budapest. Chlorpromazine was purchased from the Sigma Chemical Co., St. Louis, MO. A pure Montmorillonite Clay, "Mineral Colloid BP" (Southern Clay Products, Gonzales, TX), was employed.

Hemolytic activity was measured by a procedure

‡ Corresponding author: Dr. Paul B. Weisz, Foxdale Village, Apt. A-1, 500 East Marylyn Ave., State College, PA 16801-6236. Tel. (814) 237-3160; FAX (814) 237-3202.

‖ Abbreviations: CDS, cyclodextrin polysulfates; β-CD, β-cyclodextrin; β-CD-4Pr, 2-hydroxypropyl-β-CD (*ca.* 4 substituents/molecule); β-CD-14S, β-CD-tetradecasulfate sodium (*ca.* 14 substituents/molecule, i.e. 2/glucose unit); β-CD-14M, heptakis-(2,6-*O*-methyl)-β-CD, also known as DIMEB (β-CD with 14 methyl substituents/molecule, i.e. 2/glucose unit); and CPZ, chlorpromazine.

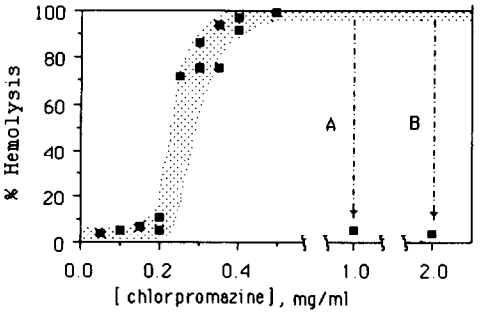


Fig. 1. Observed dependence of hemolysis on chlorpromazine concentrations between 0 and 0.5 mg/mL, and the quenching of its hemolysis by 50 mg/mL (A) and 80 mg/mL (B) of β-cyclodextrin sulfate at chlorpromazine concentrations of 1.0 and 2.0 mg/mL, respectively.

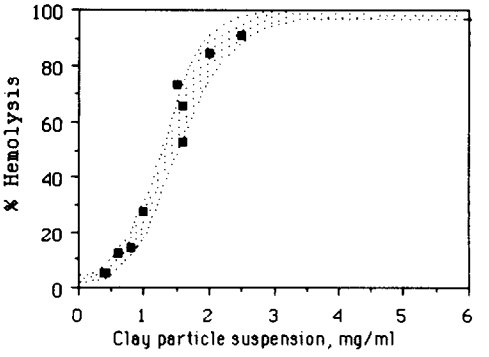


Fig. 2. Hemolysis by a Montmorillonite Clay at increasing concentrations of the suspended particles.

closely patterned after that of Jodál *et al.* [20] and previously used by us [6]. Sodium citrate was added (0.47 g/100 mL) to fresh human blood collected from healthy laboratory personnel. The supernatant of the erythrocyte fraction after centrifugation at 400 g for 10 min was washed twice with isotonic phosphate buffer, and diluted with buffer to 10% hematocrit value. Test material solution (3.6, 1.8 and 0.9 mL) was mixed with 0.1 mL of erythrocyte suspension, incubated for 15 min at 37°, and then centrifuged twice for 2 min at 4000 *g*. The supernatant was measured for absorbance at 543 nm using a Gilford spectrophotometer. The degree of hemolysis was determined by reference to that observed in samples containing saline solution only, and to that obtained after sonicating to provide a value for 100% hemolysis. The agents tested for hemolysis were all water soluble at the concentrations used, with the exception of the clay, which upon agitation was used as a colloidal suspension, with an average particle size in the range of 1 to 10 μm.

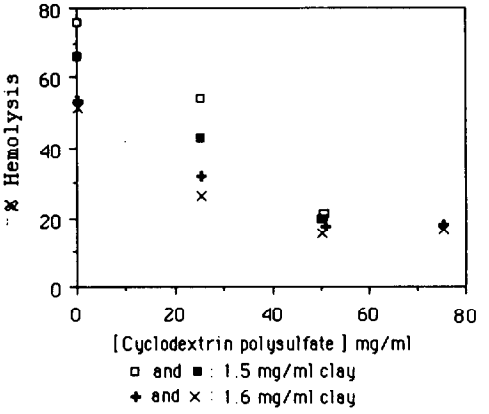


Fig. 3. Inhibition of hemolysis by increasing amounts of added CDS in several samples of blood with 1.5 to 1.6 mg/mL of suspended Montmorillonite Clay particles.

RESULTS

Inhibition of chlorpromazine-induced hemolysis. Chlorpromazine induces hemolysis above a critical concentration of about 0.2 mg/mL (6×10^{-3} M). As seen by the data in Fig. 1, the rise in the subsequent concentration interval, between 0.2 and 0.3 mg/mL, was rapid, behaving like a quasi-unstable region where reproducible quantitative results are difficult to achieve.

The addition of CDS in concentrations of 50–80 mg/mL was found to inactivate hemolysis at chlorpromazine concentrations as high as 1.0 and 2.0 mg/mL, as shown by A and B in Fig. 1, respectively.

Inhibition of silicate particle-induced hemolysis. Lysis of erythrocytes is known to occur in contact with various siliceous particulates, asbestos and other mineral particles [23, 24]. Clay particle suspensions of Montmorillonite are highly hemolytically active. Figure 2 shows measurements of the degree of hemolysis obtained on a sample of our clay, at various clay concentrations. Hemolysis increased rapidly at concentrations above approximately 1 mg/mL of suspended clay. Figure 3 presents observations of the effect of the addition of various amounts of CDS to clay concentrations of 1.5 to 1.6 mg/mL. Since clay is insoluble, it exists as a suspension or dispersion. Thus, particle aggregation and homogeneity in each sample solution depend critically upon agitation and settling effects. While the exact point of rise varied for different series of runs of different clay concentrations, a consistent inhibitory effect of added CDS was found.

Inhibition of cyclodextrin-induced hemolysis. β-Cyclodextrin is the most readily available and most frequently studied cyclodextrin. Appreciable hemolytic activity by β-cyclodextrin, at 37° in isotonic solution, has been reported [6, 19, 20] to occur at concentrations of approximately 3–5 mg/mL, and at near 1 mg/mL for β-cyclodextrin with 14 methyl group substituents (DIMEB). Substitutions by hydroxyalkyl groups decrease hemolytic activity [6] of the derivative molecule.

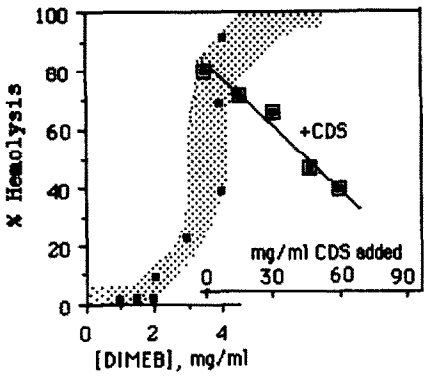


Fig. 4. Hemolysis induced by tetradecamethoxy β-cyclodextrin (DIMEB), and its deactivation by increasing amounts of added CDS, for a concentration of 3.5 mg/mL of DIMEB.

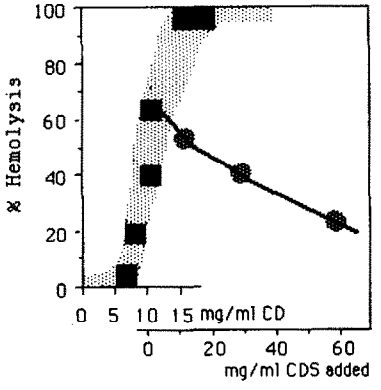


Fig. 5. Hemolysis induced by β-cyclodextrin (CD), and its deactivation by increasing amounts of CDS for a concentration of 10 mg/mL of CD.

Figure 4 shows results for hemolysis induced by DIMEB itself, i.e. β-cyclodextrin with 14 *O*-methyl groups (β-CD-14M), as a function of concentration (shaded area). Also plotted on the curve in Fig. 4 is the observed hemolysis for a fixed amount of 3 mg/mL of β-CD-14M, but with increasing amounts of CDS added (solid line).

To show that the sulfated form could suppress hemolysis induced by the unsulfated cyclodextrin, increasing amounts of the CDS were added to an erythrocyte suspension containing 6 mg/mL of cyclodextrin. Figure 5 shows the expected high degree of hemolysis of cyclodextrin, and the substantial reduction achieved by addition of CDS. Additional data shown in Fig. 6 demonstrate the intense hemolytic action of 15 mg/mL of β-CD, and the protection provided by the addition of sulfated CD.

Similar results of protection by CDS were obtained with the more soluble, and relatively less hemolytic

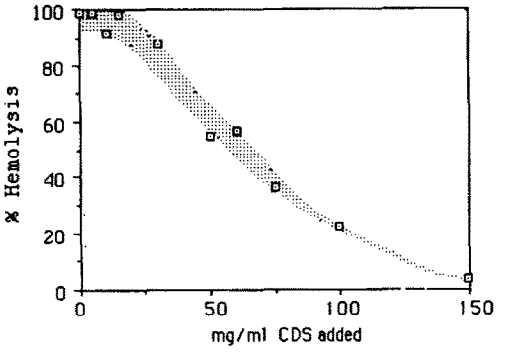


Fig. 6. Quenching of hemolysis induced by 15 mg/mL of β-cyclodextrin with increasing amounts of added CDS.

Table 1. Inhibition of the hemolytic activity of 70 mg/mL of β-CD-4Pr upon the addition of β-cyclodextrin polysulfate (CDS)

CDS added (mg/mL)	% Hemolysis
0	64
25	43
50	20
75	7
100	4

2-hydroxypropyl cyclodextrin β-CD-4Pr, as shown by the data of Table 1.

Hemolytic activity of chlorpromazine + β-cyclodextrin. Table 2 summarizes data obtained for different pairs of concentrations of chlorpromazine and β-CD. Since complexing of chlorpromazine requires at least equal molar amounts of the β-CD, the concentrations are expressed in millimolar amounts for the purpose of relevant orientation. The data represent a total of 62 individual measurements, with multiple data averaged within each concentration pair. The bold figures represent results obtained for the indicated concentration pair, but with CDS added at a concentration of 50 mg/mL.

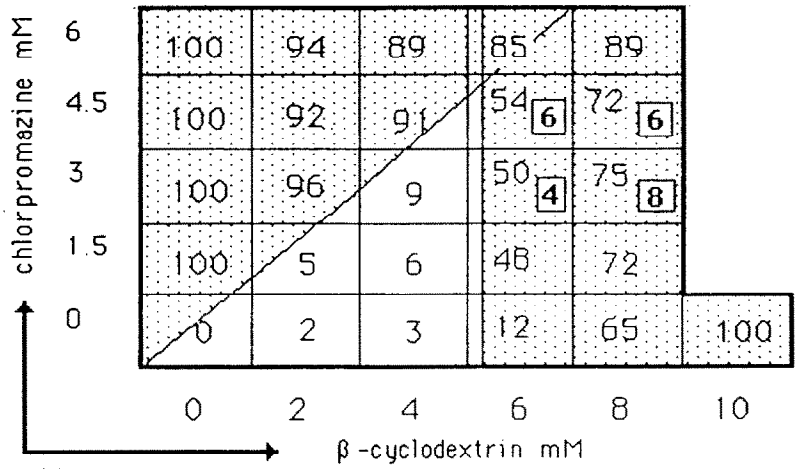
DISCUSSION

The magnitudes of hemolysis observed for each hemolytic agent used in these experiments are in good agreement with those reported previously for chlorpromazine [13–16], Montmorillonite Clay [24], and for the cyclodextrins β-CD, β-CD-14M and β-CD-4Pr [6, 19, 20]. In each case, the addition of the highly sulfated cyclodextrin β-CD-14S was found to inhibit the hemolytic effects of each and all of the hemolytic substances.

Moreover, the inhibitory effect was similar against the hemolytic agents in spite of their inherent diversity. The magnitude of CDS required for comparable deactivation was similar for all, i.e.

Table 2. Per cent of hemolysis produced in the presence of both β-cyclodextrin (β-CD) and chlorpromazine (CPZ)

% Hemolysis



The shaded area above the diagonal represents molar concentration ratios CPZ/β-CD > 1; the shaded area to the right of the vertical line represents β-CD concentrations beyond which β-CD is hemolytic. Framed and bold figures represent measurements with the addition of 50 mg/mL of CDS.

approximately 40–80 mg/mL of CDS appreciably deactivated hemolysis. This behavior, and the course of the response to increasing CDS concentration, suggest a protective interaction between the erythrocyte and CDS, such as surface adsorption of CDS on the cell membrane, with the degree of surface coverage dependent on CDS concentration in the manner of elementary Langmuir (surface science) or Michaelis–Menten (bio-science) adsorption behavior, where the fraction of maximal site occupancy η is

$$\eta = C/(1/K + C) \quad (1)$$

with C the concentration of the adsorbing agent CDS, and K the equilibrium constant for adsorption, i.e. for receptor occupancy. The remaining activity for hemolytic attack can then be considered to be proportional to the remaining fraction of unprotected

Table 3. Equilibrium constant (K) values of the anti-hemolytic stabilizing adsorptive mechanism of cyclodextrin polysulfate (CDS) on human erythrocytes

Hemolysis by	CDS (mg/mL)	$1/K$ (mg/mL)	Avg. $1/K$ (mg/mL)
Clay (1.5–1.6 mg/mL)	25	31.3	
	25	71.4	
	25	26.3	
	50	27.8	
	50	9.8	
	50	8.1	
	75	35.7	30
DIMEB (3.5 mg/mL)	15	55.6	
	30	83.3	
	45	26.3	
	60	27.8	48
CD (10 mg/mL)	10	41.7	
	30	32.3	
	60	21.3	32
CDPr (70 mg/mL)	25	30.5	
	50	14.0	22

For the observations with several of the hemolytic agents, K was calculated for each data point of concentration of added CDS, from the hemolytic conversion obtained, H, together with the respective value of hemolysis at zero added CDS, H_0, according to Equation (4). The average K thus calculated was tabulated for each hemolytic agent tested.

surface $\varepsilon = 1 - \eta$. This quantity is thus independent of the nature and agent causing the hemolytic activity k on the unprotected receptor surface,

$$k = k_0\varepsilon \quad (2)$$

where k_0 is the characteristic activity of the hemolytic agent at its prevailing concentration.

We measure hemolysis in terms of a conversion, H, i.e. a fraction of total possible conversion to free hemoglobin,

$$H = 1 - e^{-k} = 1 - e^{-k_0\varepsilon} \quad (3)$$

Using (1), (2) and (3), we can obtain the equilibrium constant K from

$$K = (1/C)\left[\frac{\ln(1 - H_0)}{\ln(1 - H)} - 1\right] \quad (4)$$

We calculated the magnitude of K from all data available for varying CDS concentrations (C) (Figs. 3–5, and Table 1), where measured hydrolysis was not less than 10% ($H < 0.9$) and not more than 90% ($H_0 > 0.1$) at $C = 0$, since the functional form becomes inaccurate and insensitive near 0 and 100% conversion. The results are presented in Table 3. The magnitude of K was similar for all cases studied. $1/K$ was approximately 20–50 mg/mL or about $8–20 \times 10^{-3}$ M of β-cyclodextrin tetradecasulfate.

The utility of β-cyclodextrin by itself as a protecting agent against chlorpromazine hemolysis by virtue of internal complexation is well illustrated by the data in Table 2, for sufficiently small molar quantities of the two materials employed. Note that, indeed, the reduction of hemolysis was observed for a limited range over which the molar ratio of β-cyclodextrin/chlorpromazine was near or above one, as expected by virtue of the molecular complexation mechanism which is involved. However, at higher total concentrations, where the β-cyclodextrin molecules themselves were hemolytically active, this mechanism of protection failed. However, the addition of CDS provided protection. It thereby extended the total amount of solubilization (complexation) that could be achieved by added (unsulfated) cyclodextrin. It is possible that CDS may itself provide an ability for additional internal complexation. However, the rate for such complex formation may be sterically hindered by the multiple sulfate groups located at the "entrance" of the cyclodextrin cavity.

While details of the molecular mechanism of stabilization by adsorbed cyclodextrin polysulfate cannot be inferred by these observations alone, it appears plausible to assume that electrostatic bonding of the multiple sulfate groups of the cyclodextrin polysulfate with cationic sites of membrane constituents stabilizes structures of large portions of the plasma membrane.

Acknowledgements—This research was supported by a grant for interdisciplinary research from the Mobil Foundation and Public Health Service Grant HL34005.

REFERENCES

1. Folkman J and Weisz PB, Interdisciplinary challenges: Control of angiogenesis. In: *Biocatalysis and Biomimetics* (Eds. Burrington JD and Clark D), American Chemical Society Symposium Series 392, pp. 19–32. American Chemical Society, Washington, DC, 1988.
2. Folkman J, Weisz PB, Joullié MM, Li WW and Ewing WR, Control of angiogenesis with synthetic heparin substitutes. *Science* **243**: 1490–1493, 1989.
3. Shing Y, Folkman J, Weisz PB, Joullié MM and Ewing R, Affinity of fibroblast growth factor for β-cyclodextrin. *Anal Biochem* **185**: 108–111, 1990.
4. Weiner DB, Williams WV, Weisz PB and Greene MI, Synthetic cyclodextrin derivatives inhibit HIV infection *in vitro*. *Pathobiology* **60**: 206–211, 1992.
5. Weisz PB, Herrmann HC, Joullié MM, Kumor K, Levine EM, Macarak EJ and Weiner D, Angiogenesis and GAG-mimics. In: *Angiogenesis—Key Principles-Science-Technology-Medicine* (Eds. Steiner R, Weisz PB and Langer R). Birkhaeuser, Basel, 1992.
6. Macarak EJ, Kumor K and Weisz PB, Sulfation and hemolytic activity of cyclodextrin. *Biochem Pharmacol* **42**: 1502–1603, 1991.
7. Sänger W, Cyclodextrin inclusion compounds in research and industry. *Angew Chem Int Ed Engl* **19**: 344–362, 1980.
8. Szejtli J, *Cyclodextrins and their Inclusion Complexes*. Akademiai Kiado, Budapest, 1982.
9. Huber O and Szejtli J (Eds.), *Advances in Inclusion Science, Proceedings of the Fourth International Symposium on Cyclodextrins*. Kluwer Academic Publishers, Boston, 1988.
10. Duchene D (Ed.), *Cyclodextrins and Their Industrial Use*. Editions de Santé, Paris, 1987.
11. Pitha J, Harman SM and Michel ME, Hydrophilic cyclodextrin derivatives enable effective oral administration of steroidal hormones. *J Pharm Sci* **75**: 165–167, 1986.
12. Friedman RB, A chemically modified cyclodextrin. In: *Advances in Inclusion Science, Proceedings of the Fourth International Symposium on Cyclodextrins* (Eds. Huber O and Szejtli J), pp. 103–111. Kluwer Academic Publishers, Boston, 1988.
13. Otagiri M, Uekama K, Irie T, Sunada M, Miyata T and Kasé Y, Effects of cyclodextrins on the hemolysis induced with phenothiazine neuroleptics. In: *Proceedings of the First International Symposium on Cyclodextrins* (Ed. Szejtli J), pp. 389–398. D. Reidel Publishing, Dordrecht, Holland, 1982.
14. Uekama K, Irie T, Sunada M, Otagiri M, Iwasaki K, Okano Y, Miyata T and Kase Y, Effects of cyclodextrins on chlorpromazine-induced haemolysis and central nervous system responses. *J Pharm Pharmacol* **33**: 707–710, 1981.
15. Uekama K, Irie T, Sunada M, Otagiri M and Tsubaki K, Protective effects of cyclodextrins on drug-induced hemolysis *in vitro*. *J Pharmacobiodyn* **4**: 142–144, 1981.
16. Irie T, Sunada M, Otagiri M and Uekama K, Protective mechanism of β-cyclodextrin for the hemolysis induced with phenothiazine neuroleptics *in vitro*. *J Pharmacobiodyn* **6**: 408–414, 1983.
17. Masuda K, Ito A, Ikari T and Terashima A, Protective effects of cyclodextrin for the local irritation induced by aqueous preparations of flurbiprofen. *Yakugaku Zasshi* **104**: 1075–1082, 1984.
18. Hoshino T, Hirayama F, Uekama K and Yamasaki M, Reduction of protriptyline-photosensitized hemolysis by β-cyclodextrin complexations. *Int J Pharm* **50**: 45–52, 1989.
19. Irie T, Otagiri M, Sunada M, Uekama K, Ohtani Y, Yamada Y and Sugiyama Y, Cyclodextrin-induced hemolysis and shape changes of human erythrocytes *in vitro*. *J Pharmacobiodyn* **5**: 741–744, 1982.
20. Jodál Y, Nánási P and Szejtli J, Investigation of the hemolytic effect of the cyclodextrin derivatives. In: *Advances in Inclusion Science, Proceedings of the*

Fourth International Symposium on Cyclodextrins (Eds. Huber O and Szejtli J), pp. 421–424. Kluwer Academic Publishers, Boston, 1988.

21. Okada Y, Kubota Y, Koizumi K, Hizukuri S, Ohfuji T and Ogata K, Some properties and the inclusion behavior of branched cyclodextrins. *Chem Pharm Bull (Tokyo)* **36**: 2176–2185, 1988.
22. Yamamoto M, Yoshida A, Hirayama F and Uekama K, Some physicochemical properties of branched beta-cyclodextrins and their inclusion characteristics. *Int J Pharm* **49**: 163–171, 1989.
23. Macnab G and Harington JS, Haemolytic activity of asbestos and other mineral dusts. *Nature* **214**: 522–523, 1967.
24. Oscarson DW, Van Scoyoc GE and Ahlrichs AJ, Lysis of erythrocytes by silicate minerals. *Clays Clay Minerals* **34**: 74–80, 1986.