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Reduction of protriptyline-photosensitized hemolysis by β-cyclodextrin complexations

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Summary

β-Cyclodextrin (β-CyD) and heptakis (2,6-di-*O*-methyl-β-CyD (DM-β-CyD)) were found to suppress the protriptyline (PTL)-induced hemolysis of human erythrocytes. The inhibitory effect of DM-β-CyD was larger than that of β-CyD in both aerobic and anaerobic conditions. The photodegradation products of PTL in the absence and presence of DM-β-CyD were analyzed by liquid chromatography/mass spectroscopy and their hemolytic activities were surveyed. The results indicate that the protective effects of β-CyDs may be due to the deceleration of the photodegradation of PTL, together with the suppression of the formation of highly toxic products such as 10,11-epoxide and cyclobutyl dimer. The alternative photoreaction pathway of PTL may result from the partial inclusion of PTL molecule within the hydrophobic cavity of β-CyDs, which was assessed by various spectroscopic methods.

Introduction

Protriptyline hydrochloride (PTL; *N*-methyl-5H-dibenzo[a,d]cycloheptene-5-propylamine), a tricyclic antidepressant, is known to cause cutaneous photosensitivity in man resulting in the serious skin-irritation (Magnus, 1976): the cell membrane is the main site of the damage (Kochevar and Lamola, 1979; Kochevar, 1980; Kochevar, 1981). In a preliminary study, we reported that the photosensitized skin irritation associated with PTL in guinea pigs was markedly alleviated by inclusion complexation with β-cyclodextrin (β-CyD) and heptakis(2,6-di-O-methyl)-β-CyD (DM-β-CyD) (Hoshino et al., 1987). In this study, the effect of β-CyD and DM-β-CyD on the PTL-photosensitized hemolysis of human erythrocytes, as a simple in vitro membrane model (Rothman, 1977), was investigated and the alleviating mechanism of β-CyDs for the photosensitized hemolysis was discussed on the basis of changes in the photochemical reactivity of PTL when complexed by β-CyDs.

Materials and Methods

Materials

PTL was supplied by Merck (F.R.G.). β-CyD and DM-β-CyD were purchased from Nippon

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Shokuhin Kako Co. (Tokyo, Japan) and Toshin Chemical Co. (Tokyo, Japan), respectively, and recrystallized twice from water. All other materials and solvents were of analytical reagent grade. Deionized double-distilled water was used throughout the study.

Photoirradiation

The light source was a sunlamp (Toshiba FL20SE30, Tokyo, Japan) which emits between 290–320 nm, with a maximum at 305 nm (mainly UVB). The energy intensity of sunlamp was approximately 7.0 mW/cm^2 at 305 nm (Radiometer UVR-305/365, Eisai Co., Japan). The test solutions containing PTL (0.5 mM) in isotonic phosphate buffer (pH 7.4) were irradiated under aerobic and anaerobic conditions through a pyrex filter. Deoxygenation of the test solutions was accomplished by purging with nitrogen for 20 min, according to the method of Gasparro and Kochevar (1982).

Photohemolysis

Human erythrocytes from freshly drawn blood were supplied by the Kumamoto Prefectural Red Cross Blood Center, Japan. Erythrocytes separated by centrifugation (1000 *g* for 10 min) were washed 3 times with isotonic phosphate buffer (pH 7.4) and then resuspended in the buffer solution to give the hematocrit value of 5%. A 0.4 ml of the erythrocyte suspension was added to 4 ml of preirradiated PTL solution. The mixture was incubated for 30 min at 37 ° C and then centrifuged at 1000 *g* for 5 min and the optical density of the supernatant was measured for hemoglobin at 588 nm. Results were expressed as % total hemolysis by comparison with a sample of the complete hemolysis in water.

Photodegradation

At predetermined times, the photoirradiated PTL solution (6 ml) was made basic with 0.1 N NaOH (1 ml) and extracted with 5 ml of ether. After evaporation of the ether, the residue was redissolved in methanol (1 ml) and subjected to high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS) for determination of PTL and its photoproducts. In analytical studies, HPLC was performed under the following conditions; pump and detector: Jasco BIP-I machine with UVIDEC-100-V UV monitor (Tokyo, Japan), column: Whatmann Particil (4.6 × 250 mm, USA), mobile phase: methanol/chloroform (3/2) containing 0.5 v/v % *n*-propylamine, flow rate: 0.8 ml/min, detection: 290 nm, internal standard: imipramine. LC/MS was performed using a Hitachi Model M-80LCAPI Mass spectrometer (Tokyo, Japan) under the following conditions; pump; Hitachi 655A machine, column: Hitachi #3056 ODS (4.0 × 150 mm), mobile phase: acetonitrile/0.05M ammonium acetate buffer at pH 4.2 (4/6), flow rate: 1 ml/min, temperature of vaporizer: 400 ° C temperature of desolvator: 400 ° C, drift voltage: 100 V. In a preparative study of photodegradation products, HPLC was performed under the conditions similar to that of LC/MS study (column: Hitachi #3056 ODS (10.0 × 150 mm), flow rate: 3.3 ml/min). The 10 fractions were collected every 2 min using a Fraction Collector (Pharmacia Fine Chemicals FRAC-100, Tokyo, Japan).

Spectroscopic studies

The UV (Hitachi 556S, Tokyo, Japan), CD (Jasco J-50A, Japan) and fluorescence (Hitachi MPF-3, Tokyo, Japan) spectra were taken at 25 ° C in an isotonic phosphate buffer (pH 7.4). ^{13}C-NMR (JEOL JNM-FX 200, Tokyo, Japan) spectra were measured at 20 ° C in D_2O, where the concentrations of PTL and β-CyDs were 0.02 M. The ^{13}C chemical shifts were recorded with an accuracy of 0.024 ppm using tetramethylsilane as an external reference. The ^{13}C-NMR peaks of PTL were assigned according to Craik et al. (1987) and by a decoupling experiment.

Results and Discussion

Effects of β-CyDs on PTL-photoinduced hemolysis

The PTL-photoinduced hemolysis is known to be caused mainly by the formation of stable photoproducts with high hemolytic activity (Gasparro and Kochevar, 1982). Therefore, PTL solutions preirradiated in the absence and presence of β-CyD or DM-β-CyD under aerobic and anaerobic

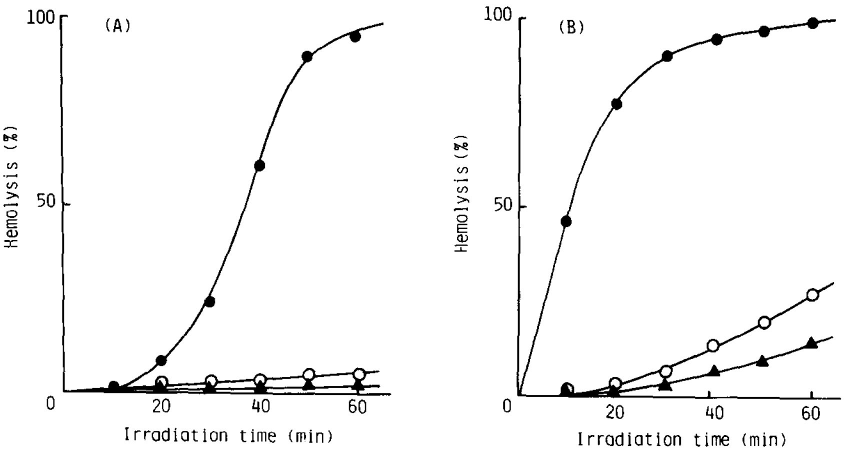


Fig. 1. Effects of β-CyDs on the hemolysis induced by photoirradiated PTL (0.5 mM) in isotonic phosphate buffer (pH 7.4). β-CyDs (0.5 mM) was added before photoirradiation. ●, PTL; ○, PTL + β-CyD; ▲, PTL + DM-β-CyD. A: irradiated under aerobic condition. B: irradiated under anaerobic condition.

conditions were used to examine their hemolytic activities against human erythrocytes. Fig. 1 shows the hemolytic activities of PTL as a function of irradiation time. The hemolytic activity of PTL solutions increased with irradiation time, and the PTL solutions irradiated under anaerobic condition showed stronger hemolysis than that irradiated under aerobic condition. These high hemolytic activities were retained after 5 days when stored in the dark at 25 ° C. It is apparent that both β-CyDs reduced the PTL-photoinduced hemolysis and the inhibitory effect was more pronounced for the DM-β-CyD system than for the β-CyD system as shown in Fig. 2. When β-CyDs were added after the irradiation of PTL solutions, however, the remarkable inhibitory effects of β-CyDs were no longer observed under aerobic condition (Fig. 3). Since β-CyDs themselves had little protective effect on the hemolysis under the experimental conditions (Irie et al., 1982), the inhibitory effect of β-CyDs may be mainly due to the alteration in the photochemical reactivity of PTL through inclusion

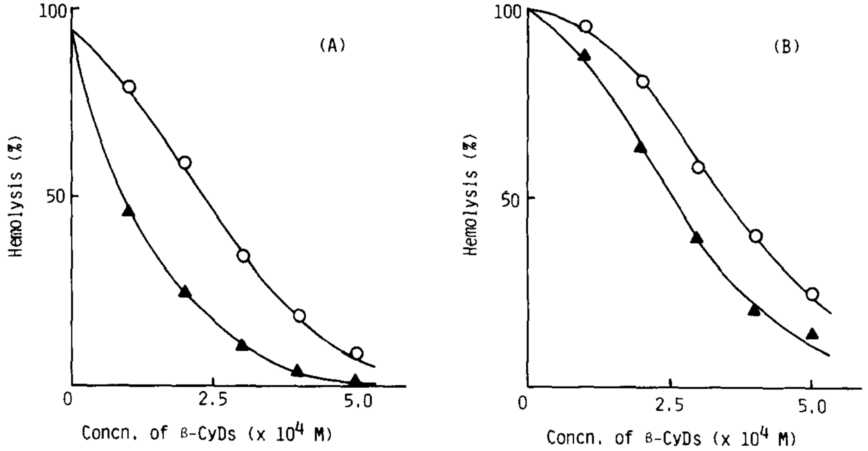


Fig. 2. Effects of β-CyDs concentrations on the hemolysis induced by photoirradiated PTL (0.5 mM) in isotonic phosphate buffer (pH 7.4). β-CyDs was added before photoirradiation. ○, PTL + β-CyD; ▲, PTL + DM-β-CyD. A: irradiated for 60 min under aerobic condition. B: irradiated for 60 min under anaerobic condition.

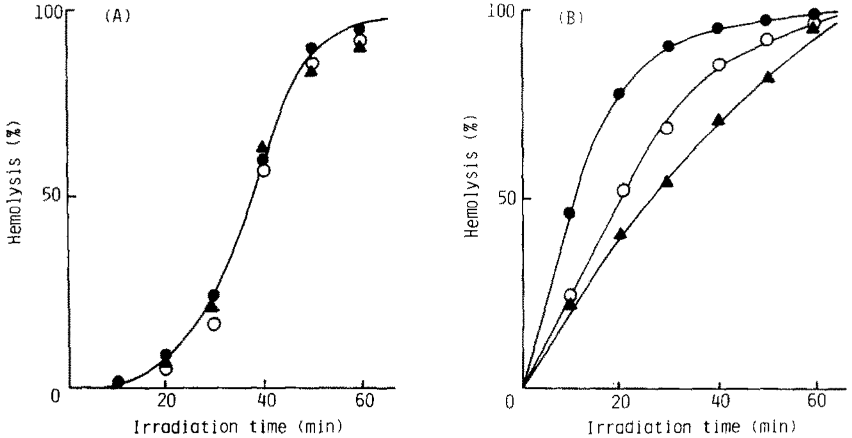


Fig. 3. Effects of β-CyDs on the hemolysis induced by photoirradiated PTL (0.5 mM) in isotonic phosphate buffer (pH 7.4). β-CyDs (0.5 mM) was added after photoirradiation. ●, PTL; ○, PTL + β-CyD; ▲, PTL + DM-β-CyD. A: irradiated under aerobic condition. B: irradiated under anaerobic condition.

complexation rather than to the direct interaction with PTL photoproducts with β-CyDs. The former mechanism seems to be predominantly involved in the inhibition under the anaerobic condition, although the latter mechanism is at least partly operative (compare Fig. 3B with Fig. 1B). Therefore, the photochemical reactivity of PTL-β-CyD complexes was investigated in the following study.

Effects of β-CyDs on photochemical reactivity of PTL

Fig. 4 shows the time courses of the photodegradation of PTL in the absence and presence of β-CyDs under aerobic and anaerobic conditions. DM-β-CyD largely decelerated the photodegradation of PTL, compared with β-CyD, suggesting that the inhibitory effect of β-CyDs on the PTL-photoinduced hemolysis is at least partly attributable to the deceleration rate in the PTL degradation. However, it is important to note that the alleviation by β-CyDs in the hemolysis was much greater than that estimated from the decelerative effect of β-CyDs. This suggests that β-CyDs may alter the photodegradation pathway of PTL to suppress the formation of highly toxic products. Therefore, the photoproducts of PTL were

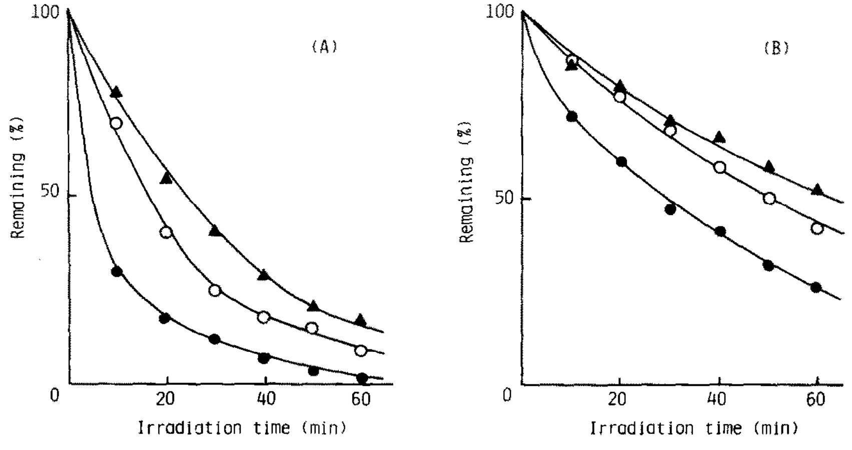


Fig. 4. Effects of β-CyDs (0.5 mM) on the photodegradation of PTL (0.5 mM) in isotonic phosphate buffer (pH 7.4). ●, PTL; ○, PTL + β-CyD; ▲, PTL + DM-β-CyD. A, under aerobic condition. B, under anaerobic condition.

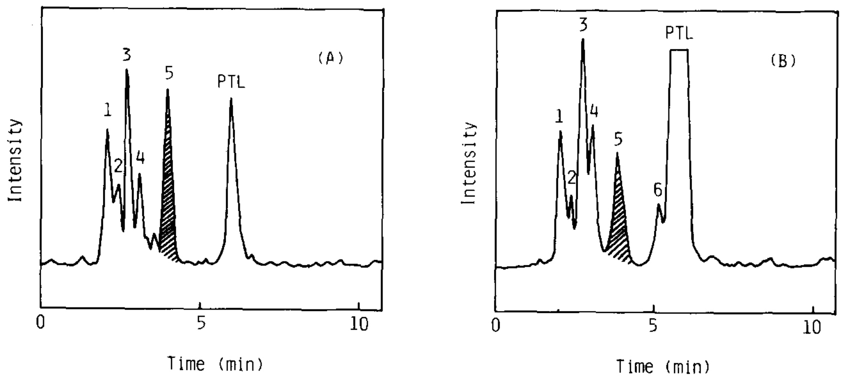


Fig. 5. LC/MS total ion chromatograms of PTL (0.5 mM) photoproducts formed in the absence and presence of DM-β-CyD (0.5 mM) under aerobic condition. A: irradiated for 60 min in the absence of DM-β-CyD. B: irradiated for 60 min in the presence of DM-β-CyD. The hemolytic activity of the corresponding fraction was illustrated in the shaded line.

analyzed by a liquid chromatography/mass spectroscopy (LC/MS) and their hemolytic activities were compared. Figs. 5 and 6 show the LC/MS total ion chromatograms of PTL photoproducts formed in the absence and presence of DM-β-CyD under aerobic and anaerobic conditions, respectively. The hemolytic activities of the fractions corresponding to each peak in Figs. 5 and 6 were also measured, as illustrated in shaded lines. Main peaks in the chromatograms were identified as listed in Table 1, by mass spectral analysis, i.e. m/z and fragmentation (Jones and Sharples, 1984). It is apparent that the photochemical behavior of PTL is different under the aerobic and anaerobic conditions. On irradiation with oxygen (Fig. 5), PTL was oxidized to the 10,11-epoxide and various hydroxylated products, where the fraction containing epoxide had extremely high hemolytic activity as reported by Jones and Sharples (1984). This epoxide formation was suppressed by the addition of DM-β-CyD, while formations of hydroxylated PTL (peak 3 and 4) and desmethyl-PTL (peak 6) with weaker hemolytic activity were accelerated (Fig. 5B). In the case of anaerobic condition (Fig. 6), the cyclobutyl dimer of PTL (peak 7) (Kopecky, 1971), desmethyl-PTL and other hydroxylated PTL were formed as main products, where the cyclobutyl dimer had considerably high hemolytic activity than other products as reported by Gasparro and Kochevar (1982). In the presence of DM-β-CyD (Fig. 6B), the dimerization of PTL was effectively suppressed, leading

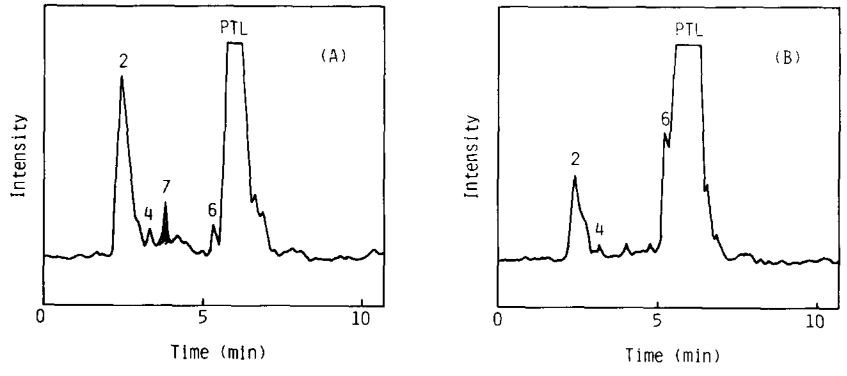
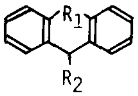
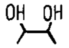
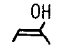
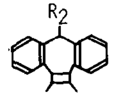


Fig. 6. LC/MS total ion chromatograms of PTL (0.5 mM) photoproducts formed in the absence and presence of DM-β-CyD (0.5 mM) under anaerobic condition. A: irradiated for 60 min in the absence of DM-β-CyD. B: irradiated for 60 min in the presence of DM-β-CyD. The hemolytic activity of the corresponding fraction was illustrated in the shaded line.

TABLE 1

Proposed structures of PTL Photoproducts



Peak	M.W.	R_1	R_2
1	297		$-CH_2CH_2CH_2NHCH_3$
2	281	unknown	unknown
3	295	unknown	unknown
4	279		$-CH_2CH_2CH_2NHCH_3$
5 *	279		$-CH_2CH_2CH_2NHCH_3$
6	249		$-CH_2CH_2CH_2NH_2$
7 *	526		$-CH_2CH_2CH_2NHCH_3$

Structures are estimated by m/z and fragmentation analysis in LC/MS. * High hemolytic activity.

to the large inhibition in the PTL-photoinduced hemolysis. Similar results were obtained for the β-CyD system. Therefore, the inhibitory effect of β-CyDs on the PTL-photoinduced hemolysis may be attributable to the deceleration in the degradation of PTL, together with the alteration in the photoreaction pathway, to suppress particularly the epoxidation and dimerization of PTL. Further studies are now in progress to identify the chemical structure of hydroxylated PTL (peak 2, 3) with weaker hemolytic activity and to assay accurately the hemolytic activity of PTL-photoproducts.

Inclusion complexation of PTL with β-CyDs

To gain insight into the decelerative mechanism of β-CyDs, the inclusion mode of PTL within the cavity of β-CyDs in aqueous solutions was investigated. Fig. 7 shows the ultraviolet (UV), circular dichroism (CD) and fluorescence spectra of PTL in the absence and presence of β-CyDs in phosphate buffer (pH 7.4). The optical activity of PTL was induced with negative and positive signs at about 240 and 290 nm, respectively, by the binding to β-CyDs. The UV absorbance of PTL at 290 nm decreased by the addition of β-CyDs, whereas its fluorescence intensity increased. Similar UV and fluorescence spectral changes were observed when PTL was dissolved in less polar solvents such as ethanol and dioxane (Uekama et al., 1978), suggesting a location of the drug chromophore within the hydrophobic and asymmetric

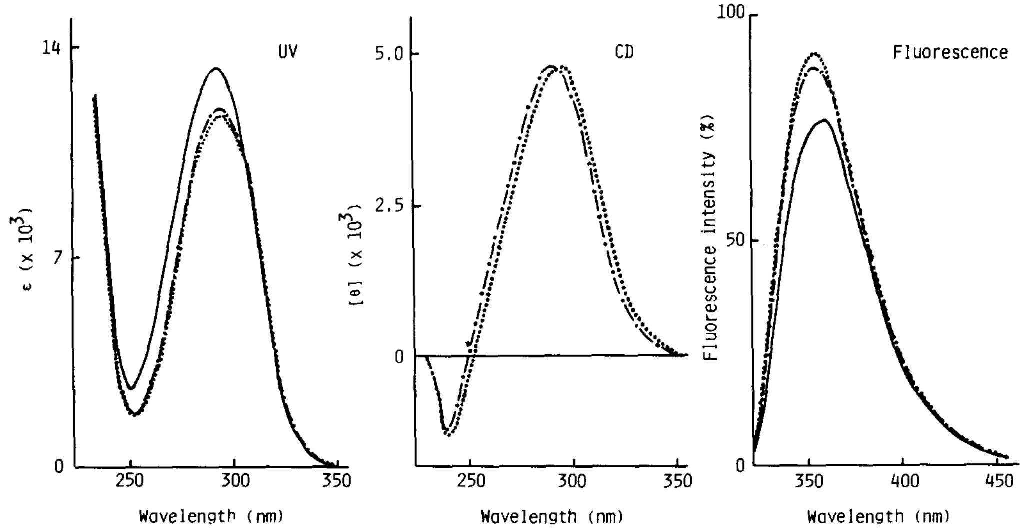
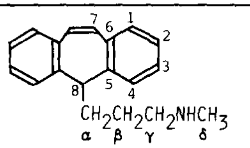


Fig. 7. UV, CD and fluorescence spectra of PTL in the absence and presence of β-CyDs in isotonic phosphate buffer (pH 7.4). ——: PTL, -·-·-: PTL + β-CyD, ······: PTL + DM-β-CyD.

TABLE 2

^{13}C-NMR chemical shift changes (ppm) of PTL (2×10^{-2} M) following the binding to β-CyDs (2×10^{-2} M) in D_2O.



Carbon	β-CyD	DM-β-CyD
1	−0.20	−0.33
2	−0.52, −0.43	−0.58, −0.42
3	−0.33	−0.55, −0.35
4	−0.04, 0.13	−0.14, 0.17
5	−0.07	−0.16
6	−0.30, −0.14	−0.38, −0.23
7	0.17	0.17
8	0.12	0.12
α	0.56	0.50
β	0.85	0.82
γ	1.63	1.73
δ	0.07	−0.02

A chemical shift change is expressed as ($\delta_{CyD} - \delta_0$) in ppm.

locus of β-CyD cavities. Since the isolation of solid PTL-β-CyD complexes was difficult due to their high aqueous solubility, the stoichiometry of both complexes was investigated by the continuous variation method (Job, 1928) of the molar ellipticity changes in CD spectra and estimated to be 1 : 1. The stability constants were determined to be 14200 M^{-1} and 17300 M^{-1} for the β-CyD and DM-β-CyD complexes, respectively, by Scott plots (Scott, 1956) of the UV spectral changes in Fig. 7.

The ^{13}C-NMR technique was employed to assess the inclusion mode of PTL within β-CyD cavities. The ^{13}C-NMR spectrum of PTL consisted of 12 peaks assigned to the magnetically non-equivalent carbons. By the binding to β-CyDs, however, the peaks for the equivalent carbons in the two aromatic rings (C_2, C_3, C_4 and C_6, see Table 2) were shifted apart suggesting that one of the aromatic rings of PTL is located inside and one outside the β-CyD cavity, i.e. magnetically different environments. Table 2 summarized the β-CyDs induced ^{13}C-chemical shift changes of PTL in D_2O. The carbons in the aromatic ring of PTL showed upfield shifts, which may be due to hydrophobic interaction with the β-CyD cavity (Conover et al., 1974). On the other hand, the aliphatic side chain of PTL may be fixed, in an extended conformation, around the hydrophilic hydroxyl group of β-CyD rims (Mason et al., 1986; Munro et al., 1986), because of the large downfield shifts. The shifts were greater in the DM-β-CyD complex than in the β-CyD complex due to the larger stability constants. These results suggest that the reactive site of PTL, i.e. C_7 is sterically shielded by the partial inclusion within the hydrophobic cavity of β-CyDs, which may consequently provide the alteration in photoreactivity of PTL.

Although the detailed mechanism of the alteration by β-CyDs in photodegradation of PTL should be investigated further, the present data indicate that β-CyDs, particularly DM-β-CyD, is useful in alleviating the photosensitized membrane damage caused by PTL, from the safety point of view (Uekama and Otagiri, 1987).

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References

Conover, W.W., Fried, J., Carbon-13 relaxation and proton nuclear magnetic resonance studies of prostaglandin $F_{2\alpha}$. *Proc. Nat. Acad. Sci. U.S.A.*, 71 (1974) 2157–2161.

Craik, D.J., Hall, J.G. and Munro L.A., Two-dimensional magnetic resonance spectra of selected tricyclic antidepressants. *Chem. Pharm. Bull.*, 35 (1987) 188–194.

Gasparro, F.P. and Kochevar, I.E., Investigation of protriptyline photoproducts which cause cell membrane disruption. *Photochem. Photobiol.*, 35 (1982) 351–358.

Hoshino, T., Ishida, K., Irie, T., Hirayama, F and Uekama, K., Alleviation in protriptyline-photosensitized skin irritation by di-*O*-methyl-β-cyclodextrin complexation. *Int. J. Pharm.*, 38 (1987) 265–267.

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. Pharm. Dyn.*, 5 (1982) 741–744.

Job, P., Formation and stability of inorganic complexes in solution. *Ann. Chim.*, 9 (1928) 113–203.

Jones, G.E. and Sharples, D., Structure elucidation of protriptyline photoirradiation products. *J. Pharm. Pharmacol.*, 36 (1984) 46–48.

Kochevar, I.E., Possible mechanisms of toxicity due to photochemical products of protriptyline. *Toxicol. Appl. Pharmacol.*, 54 (1980) 258–264.

Kochevar, I.E., Phototoxicity mechanisms: chlorpromazine photosensitized damage to DNA and cell membranes. *J. Invest. Dermatol.*, 77 (1981) 59–64.

Kochevar, I.E. and Lamola, A.A., Chlorpromazine and proyriptyline phototoxicity: Photosensitized, oxygen-independent red cell hemolysis. *Photochem. Photobiol.*, 29 (1979) 791–796.

Kopecky, J. and Shields, J.E., Photochemical cycloaddition reactions. II. Dimerization and cycloadduct formation of some seven-membered carbocycles. *Collect. Czech. Chem. Commun.*, 36 (1971) 3517–3526.

Magnus, I.A., *Dermatological Photobiology.* Blackwell, London, 1976.

Mason, N.J., Geary, W.J., Nixon, L.A. and Nowell, I.W., Interactions between phenothiazine drugs and metal ions. Part 2. Crystal and molecular structure of protonated trichloro[2-chloro-10-(3′-dimethyl-aminopropyl) phenothiazine-S]palladium(II), [Pd(cpzH)Cl_3]; hydrogen-1 and carbon-13 nuclear magnetic resonance data for the chlorpromazine complexes [M(cpzH)Cl_3](M = Pd or Pt). *J. Chem. Soc. Dalton. Trans.*, (1986) 1347–1350.

Munro, S.L., Andrews, P.R., Craik, D.J. and Gale, D.J., ^{13}C NMR studies of the molecular flexibility of antidepressants. *J. Pharm. Sci.*, 75 (1986) 133–141.

Rothman, J.E. and Lenard, J., Membrane asymmetry. *Science*, 195 (1977) 743–753.

Scott, R.L., Some comments on the Benesi-Hildebrand equation. *Rec. Trav. Chim.*, 75 (1956) 787–789.

Uekama, K., Hirayama, F. and Yamasaki, S., Micellar effects on base-catalyzed isomerization of prostaglandin A_1 and prostaglandin A_2. *Bull. Chem. Soc. Jpn.*, 51 (1978) 1229–1230.

Uekama, K. and Otagiri, M., *Cyclodextrins in Drug Carrier Systems. Vol. 3,* CRC, Boca Raton, FL, 1987, pp. 1–40.