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Effect of β-Cyclodextrin Complexation on the Photohaemolitic Activity Induced by Ketoprofen and Naproxen Sensitization

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Abstract. Red blood cell lysis photosensitized by two non-steroidal anti-inflammatory drugs Naproxen (NAP) and Ketoprofen (KPF) was investigated in the presence of β-cyclodextrin (β-Cyd). The photohaemolysis was inhibited by the addition of β-Cyd both for NAP and, to a lesser extent, for KPF. The protective action was found only in a restricted range of concentration of β-Cyd. Higher amounts of β-Cyd interfered with the resistance of the cell to the osmotic shock induced by the photosensitization process. The complexing action of β-Cyd was ascertained through UV-vis absorption spectroscopy, induced circular dichroism and emission spectroscopy.

The isolated complexes Naproxen–β-Cyd (NAP–β-Cyd) and Ketoprofen–β-Cyd (KPF–β-Cyd) were found to protect from the photosensitized membrane damage induced by the two drugs, even if it occurred only in a limited range of concentration. This suggests a valid tool in alleviating the *in vitro* phototoxic consequences caused by these compounds, even if care has to be taken in therapeutic administration due to the presence of the uncomplexed β-Cyd.

Key words: Naproxen, Ketoprofen, β-cyclodextrins, photohaemolysis, photoprotection, photosensitization.

1. Introduction

In previous studies [1,2], we have reported that the two drugs belonging to the group of non-steroidal anti-inflammatory drugs (NSAID), Naproxen (NAP) and Ketoprofen (KPF), are able to induce photohaemolysis even in low doses, in the range of concentrations attained in blood serum after therapeutical subministration.

Photohaemolytic activity induced by NAP was observed both in aerobic and anaerobic conditions and thus the membrane damage occurs by both oxygen-dependent and independent mechanisms. The results of the experiments carried

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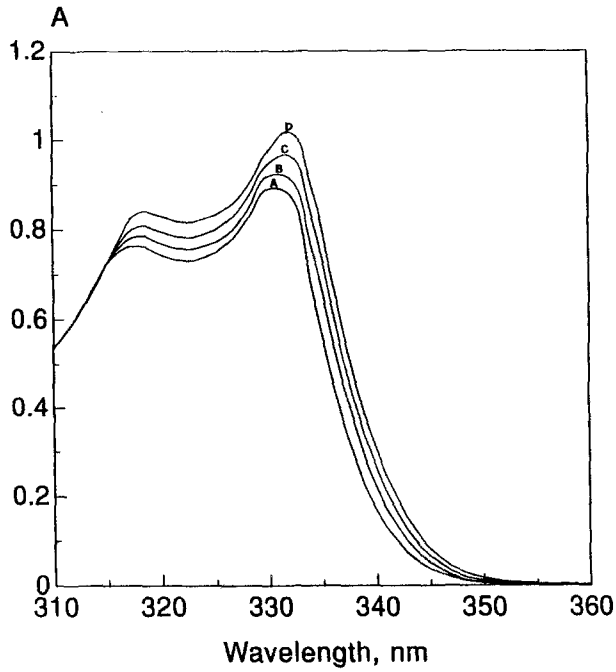

Fig. 1. Spectral changes of NAP PBS solutions (6.2×10^{-4}) in the presence of increasing amounts of β-Cyd: A = 0; B = 1; C = 2.4; D = 4.8×10^{-3} M.

out in the presence of additives suggest the involvement of free radicals, singlet oxygen and superoxide anion in the photosensitization process.

Direct photolysis of buffered solutions of NAP at pH 7.4 leads to a decarboxylation process *via* intermediate radicals, and under aerobic conditions a photooxidation leading to the photoproduct 6-methoxy-2-acetonaphthone (6-MAN) occurred. The low quantum yield of NAP photodegradation and the negligible lytic activity of the photoproduct suggest that it is not involved in the membrane damage.

On the other hand, the KPF induced photohaemolysis is strongly affected by the drug photodegradation, which occurs with a high quantum yield, and in air-saturated solutions irradiation of the drug leads to formation of the compounds (3-benzoylphenyl)ethane, (3-benzoylphenyl)ethyl hydroperoxide, (3-benzoylphenyl)-ethanol and (3-benzoylphenyl)ethanone, whereas in deaerated solution only the first compound was obtained. All the photoproducts, which are formed *via* intermediate radicals, showed lytic activity, which was particularly high for the alcohol derivative. In addition, the KPF photosensitized haemolysis involved free radicals and superoxide anion.

In this study we examined the effect of complexation between these two drugs and β-cyclodextrin (β-Cyd) on the photohaemolysis rate, with the aim of clarifying the influence of complexation on the phototoxic efficiency of the photosensitization

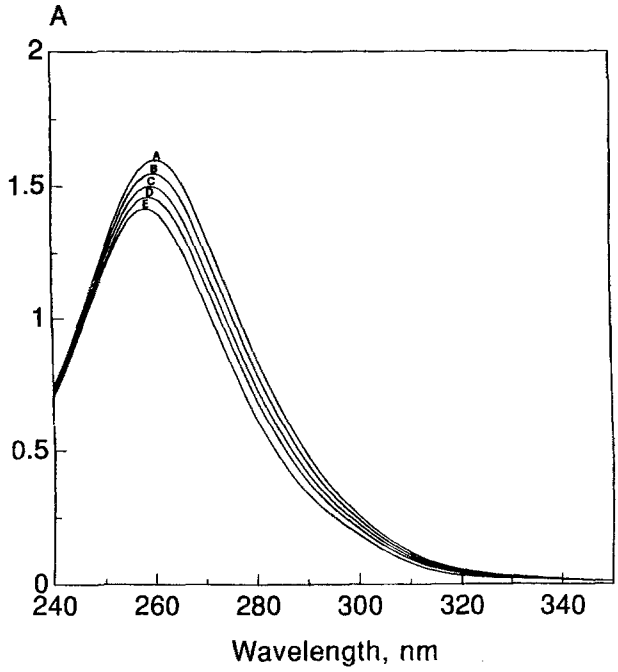

Fig. 2. Spectral changes of KPF PBS solutions (1.0×10^{-4} M) in the presence of increasing amounts of β-Cyd: A = 0; B = 0.2; C = 1.2; D = 2; E = 8×10^{-3} M.

process.

2. Experimental

2.1. Reagents

NAP, KPF, D-Glucose and β-Cyd were obtained from the Sigma Chemical Company; all other chemicals were reagent grade. Phosphate buffer saline (PBS) (pH 7.4) consists of a 0.01 M phosphate buffer and 0.135 M NaCl solution.

2.2. Irradiation conditions

Irradiation was performed with a Rayonet photochemical reactor equipped with 1–4 'black light' phosphor lamps with emission in the 310–390 nm range. A 'merry-go-round' irradiation apparatus was used to ensure that all parallel samples received equal radiation. When indicated, monochromatic radiation was obtained from a Hg Vapour Lamp (Hanau Q 700). 313 nm radiation was isolated by means of a combination of filters, consisting of 1 cm of a 0.02% solution of K_2CrO_4 in 0.05 N NaOH and 2 mm of chance glass OXI (Ealing Sc. Ltd. London).

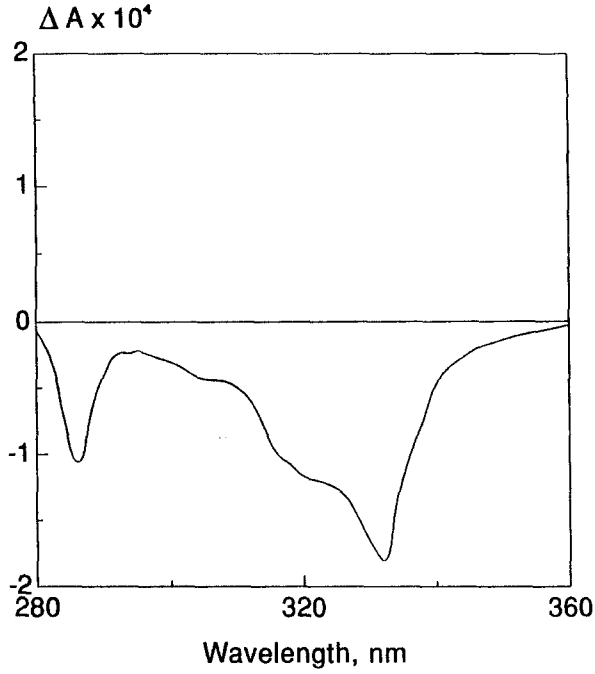

Fig. 3. Induced circular dichroism spectrum of the NAP–β-Cyd complex in PBS. [NAP] = 6.2×10^{-4} M, [β-Cyd] = 1.2×10^{-2} M.

The photon flux, incident on a 3 mL solution in quartz cuvettes of 1 cm optical path, was of the order of $10^{15} - 10^{16}$ quanta s^{-1} for the phosphor lamps and 7×10^{14} quanta s^{-1} for the mercury vapor lamp at 313 nm. The light intensity of the monochromatic radiation was measured by means of the ferric oxalate actinometer [3] and the 'black light' was measured by means of a Spectroline Model DRC-100X digital radiometer equipped with a DIX-365 sensor with a spectral range of 320–380 nm. For comparison, the measured solar fluence incident on skin was of the same order as that of the phosphor lamps.

2.3. HAEMOLYSIS ASSAYS

Red blood cells (RBC) were prepared by washing a sample of out-of-date packed human erythrocytes four times with a tenfold volume of PBS, each time centrifuging the cells at 2500 *g* for 15 min and carefully removing the supernatant.

The rate of haemolysis increases slightly with age in erythrocytes from freshly drawn blood compared with packed cells kept stored in sealed bags in a blood bank. However, erythrocytes from out-of-date packed cells give results reproducible over many days [4].

For both photohaemolysis and dark haemolysis experiments, RBC were diluted in PBS containing the drug or mixtures of drug and cyclodextrin in variable con-

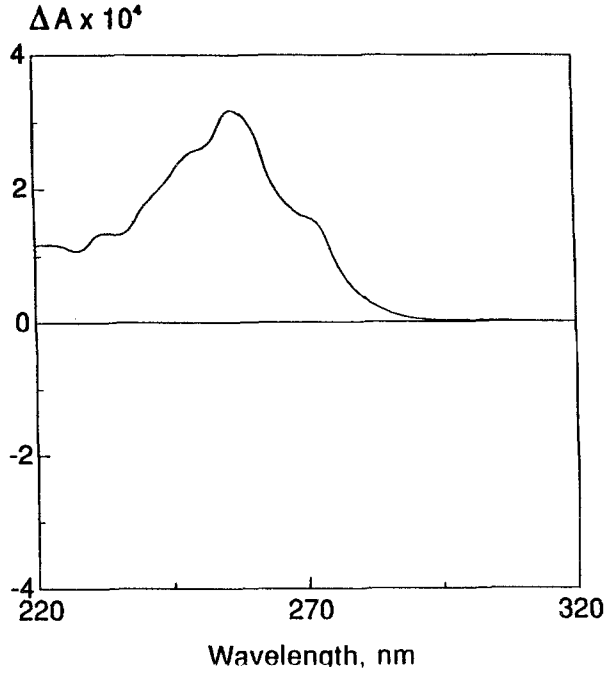

Fig. 4. Induced circular dichroism spectrum of the KPF–β-Cyd complex in PBS. [KPF] = 1×10^{-4} M, [β-Cyd] = 1.2×10^{-2} M.

centrations so that the resultant suspension had an optical density of 0.4–0.8 at 650 nm. An absorbance (A) of 0.5 corresponded to 3.3×10^6 cells/mL.

The haemolysis rate was determined by measuring the decrease in absorbance at 650 nm, since the optical density is linearly proportional to the number of intact RBC [5]. Results are expressed as a percentage of total haemolysis by comparison with a sample in which the cells had been completely haemolyzed by brief sonication.

2.4. PREPARATION OF THE COMPLEXES

The solid Naproxen–β-Cyd complex (NAP–β-Cyd) was prepared by a freeze drying method [6], by mixing β-Cyd and NAP in a 1 : 1 molecular ratio in aqueous ammonia and subsequent freeze drying. In the isolated complex NAP and cyclodextrin showed a molecular ratio of 1 : 1.

The Ketoprofen–β-Cyd (KPF–β-Cyd) inclusion complex was prepared by the coprecipitation method [7], where solutions of β-Cyd in water and KPF in methanol were mixed with a 1.5 : 1 molecular ratio, respectively. The mixture was stirred at 60°C for 1h, and then at room temperature for 24h. The complex, with KPF and β-Cyd in a 1 : 1 molar ratio, was obtained as a microcrystalline precipitate and was

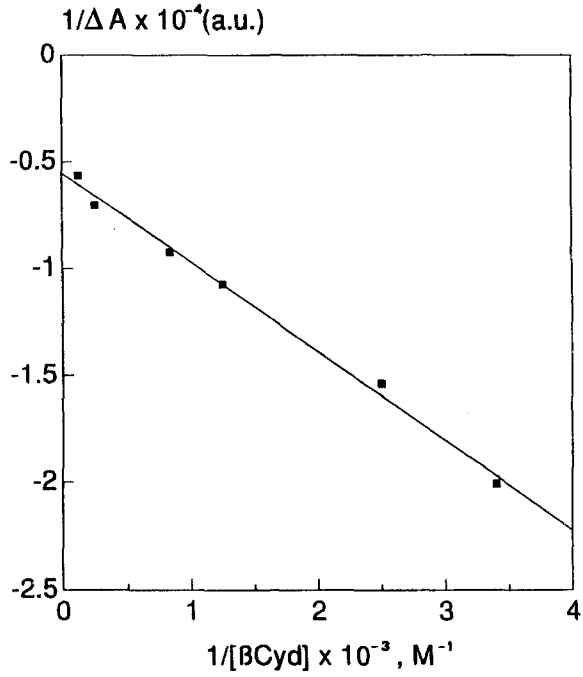

Fig. 5. Benesi–Hildebrand plot for the icd signal at 332 nm of 6.2×10^{-4} M NAP PBS solutions in the presence of various amounts of β-Cyd.

washed with water and dried overnight *in vacuo*.

The powdered inclusion compounds of the drugs with cyclodextrins were characterized according to the literature [8–12] by infrared spectroscopy, differential scanning calorimetry, and NMR.

2.5. Absorption, Emission and Circular Dicroism (ICD) Spectra

Absorption spectra were taken on a Hewlett-Packard diode array spectrophotometer model HP 8452A. Measurements of absorbance at 650 nm of red blood cells suspension were taken on a Perkin Elmer UV–vis spectrophotometer model 330. The emission spectra were taken on a Perkin Elmer spectrofluorimeter. The icd spectra were taken on a Jasco spectropolarimeter. All measurements were carried out in 0.01 M PBS pH 7.4 at 25°C.

3. Results and Discussion

3.1. Inclusion Complexation of KPF and NAP with β-Cyd

Inasmuch as the photosensitization processes applied to biological systems have to be carried out in buffered saline media at pH 7.4, the experimental approach

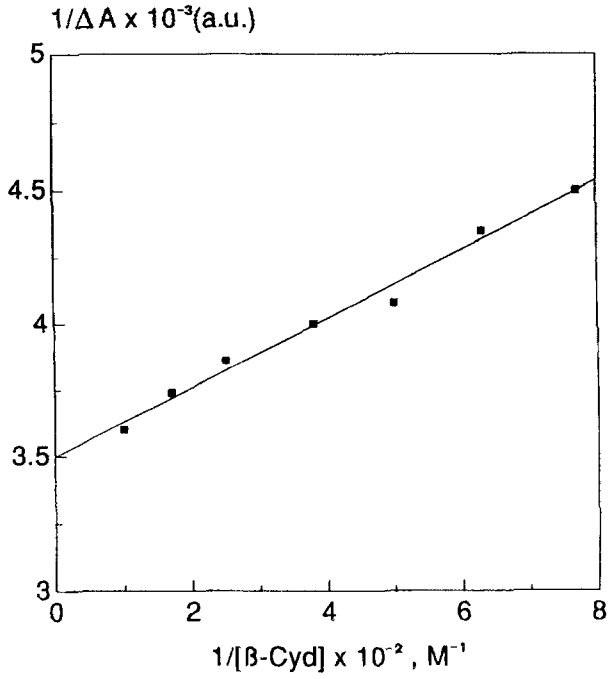

Fig. 6. Benesi–Hildebrand plot for the icd signal at 256.6 nm of 1.0×10^{-4} M KPF PBS solutions in the presence of various amounts of β-Cyd.

is directed to clarifying whether the complexation processes between the drugs considered and β-Cyd can also occur in these systems.

Inclusion was achieved by preparing mixtures of the drugs in PBS at variable concentrations of β-Cyd. For all solutions the equilibrium was obtained by stirring the mixtures in a water bath thermostatted at 25°C for at least 24 h. It was determined that this was a sufficient time to ensure equilibrium for all the substances tested.

Evidence for the formation of the inclusion complexes was achieved through: (i) UV-vis absorption spectroscopy; (ii) induced circular dichroism; and (iii) emission spectroscopy.

(i) Figures 1 and 2 show the absorption spectra of NAP and KPF in the presence of various amounts of β-Cyd in PBS. The reference used for recording these spectra consisted of buffer solution containing the same concentration of β-Cyd as that of the sample. The absorption spectra of NAP and KPF were influenced by the addition of β-Cyd. In the case of NAP the absorption maxima in the UVA region at 318 and 330 nm increased; whereas the opposite behaviour was observed for the absorption maximum of KPF at 260 nm. On the other hand, the addition of various amounts of D-glucose in place of β-Cyd, under the same experimental conditions, did not affect the absorption spectra of the two drugs: the sugar is the constituent unit making up β-cyclodextrin. The spectral changes suggest the possibility of the

formation of the inclusion complex.

(ii) In the presence of β-Cyd an induced cd spectrum of NAP and KPF appeared as seen in Figures 3 and 4, whereas it was not seen when D-glucose was added in place of β-Cyd. The icd spectrum of the system NAP/β-Cyd showed a very small positive band at wavelength shorter than 260 nm, a small negative band at 283 nm and a stronger negative band at 332 nm. For KPF, addition of cyclodextrin resulted in the formation of a very weak maximum at 256.6 nm. The icd signal was strongly affected by the β-Cyd concentration; the amounts of β-Cyd used were in the range of 1.3–20 times higher than that of NAP and 4–100 times higher than that of KPF. These results confirm the formation of inclusion complexes between β-Cyd and NAP and KPF, respectively, and this process is the origin of the Cotton effect.

The Benesi-Hildebrand plots of the icd signals for the inclusion complexes of the two drugs are shown in Figures 5 and 6: the corresponding mathematical treatment [13] as a result gave the equilibrium constants for complex formation, which for NAP and KPF are 1450 and 2700 M^{-1}, respectively. The equilibrium constants found for the β-Cyd–NAP and KPF complexes are in good agreement with the values reported for other naphthalene and benzophenone derivatives [14–16].

(iii) The intensity of the fluorescence maximum of NAP (λ_{exc} 318 nm, λ_{em} 354 nm) increased upon the addition of β-Cyd. This tendency, viz. alteration of the luminescence spectra, also suggests the formation of the inclusion complex. Unluckily the study on the influence of the inclusion effect of cyclodextrin on the luminescence of KPF was complicated by the experimental conditions, inasmuch as the weak KPF luminescence was submerged by the emission of β-Cyd.

3.2. Effect of β-Cyd on the photohaemolysis induced by Naproxen

The results of the experiments of photohaemolysis induced by Naproxen (1.4×10^{-4} M) in the presence of β-Cyd 1.65×10^{-4} M, under aerobic conditions at various times of irradiation are reported in Figure 7. The photon flux was 7×10^{15} quanta s^{-1} for 3 mL of solution. The haemolysis measurements were taken 2 h after the beginning of the irradiation and the temperature was maintained at 25°C. No lysis was observed during this time when cells were irradiated in the absence of NAP or when cells were incubated in the dark with NAP and/or β-Cyd. The results show that β-Cyd strongly reduces the haemolysis rate.

The photohaemolysis data were also obtained from experiments carried out with RBC suspensions containing NAP (1.4×10^{-4} M) in the presence of increasing amounts of β-Cyd in the concentration range of $(1 - 6) \times 10^{-4}$ M, under aerobic conditions. The irradiation time was 10 minutes and the haemolysis was followed as a function of the post-illumination time, and the time needed to produce 50% lysis, t_{50}, was determined. A 'protection factor' was calculated from the ratio between the t_{50}, with and without β-Cyd, respectively: values > 1 indicate protection. A value equal to 1 was assigned to the experiment carried out under aerobic conditions without addition of β-Cyd in the range of the concentration used and within the

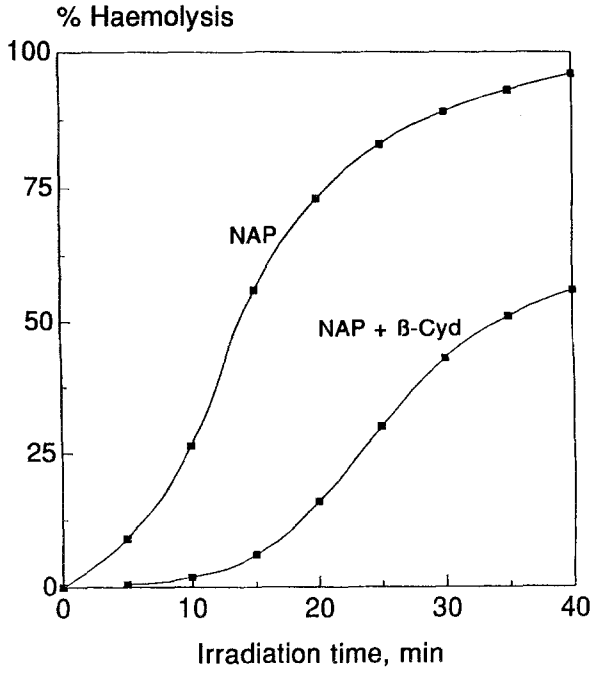

Fig. 7. Photohaemolysis of RBC sensitized by NAP in the presence or in the absence of β-Cyd. [NAP] = 1.4×10^{-4} M; [β-Cyd] = 1.65×10^{-4} M [RBC] = 3.3×10^{6} cells/mL; temperature 20°C. Each point is the mean ±SD of triplicate experiments.

time needed to observe the drug-photoinduced haemolysis (max 8 h). The results are shown in Figure 8.

Photosensitized haemolysis of RBC is indicative of membrane damage [17,18]. In this case the experimental data indicated that the damage is less extensive in the presence of β-Cyd, and consequently also in the presence of the complexed NAP. The concentration of this latter compound is reported on the Y2 axis of Figure 8. The complex concentration was calculated through the K_S value of the NAP–β-Cyd complex; a possible interaction between β-Cyd and cell membrane, was neglected in the calculation.

On the other hand, addition of amounts of cyclodextrin over 1.8×10^{-4} M, with the aim of further decreasing the concentration of free sensitizer, did not give the expected results: the 'hill profile' shows that added β-Cyd does not protect so efficiently at higher concentrations.

As a consequence it is clear that β-Cyd reduced the NAP-photoinduced haemolysis due to the probable alteration of the photosensitizer properties of the drug through inclusion complexation; this process can lead to a decrease in the concentration of transient active species such as singlet oxygen, superoxide anion and radicals, which are involved in the NAP photoinduced cell damage. On the other hand, a parallel, direct interaction of β-Cyd with the cell membrane might contrast

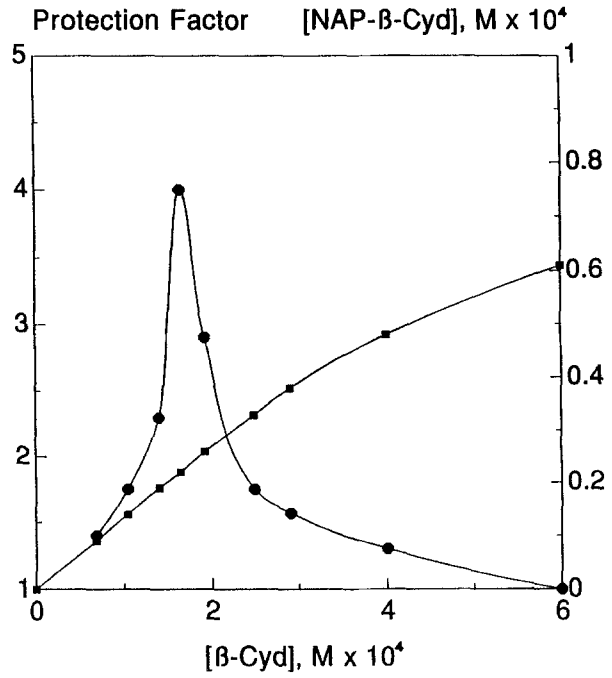

Fig. 8. Photohaemolysis of RBC sensitized by NAP in the presence of increasing amounts of β-Cyd. [NAP] = 1.4×10^{-4} M; [RBC] = 3.3×10^{6} cells/mL; irradiation time 10 min; (•) = Protection factors; (■) = [NAP–β-Cyd]; temperature 20°C. Each point is the mean ±SD of triplicate experiments.

this action, reducing its resistance to the photohaemolytic osmotic shock. In fact cyclodextrins are able, for example, to damage the lipoprotein membrane of human erythrocytes at concentrations which depend strongly on the lypophilicity of the molecule (γ-Cyd > α-Cyd > β-Cyd) [19].

The experimental data support the hypothesis that cyclodextrin in low concentrations generally does not interfere with cells, i.e. it does not show thermal lytic activity towards RBC within the time of the experimental observation. On the other hand, the lysis can be observed in the dark if RBC are incubated for a long period (more than 24 h) with β-Cyd in the concentration range $1 \times 10^{-3} - 1 \times 10^{-4}$ M. The experimental results show that this action can be amplified if the biological substrate has been previously attacked by damaging agents such as stable or transient species generated through photosensitization.

This hypothesis is also supported by an experiment carried out by irradiating aliquots of RBC suspensions in PBS solutions of NAP and then by adding increasing amounts of β-Cyd. The haemolysis rate increased with the concentration of added β-Cyd and the overall samples showed a haemolysis rate higher than the control, where β-Cyd was absent (Figure 9). In an another experiment, β-Cyd was added to aliquots of NAP/RBC suspensions which had been previously irradiated at various times. In this case the rate of delayed haemolysis increased

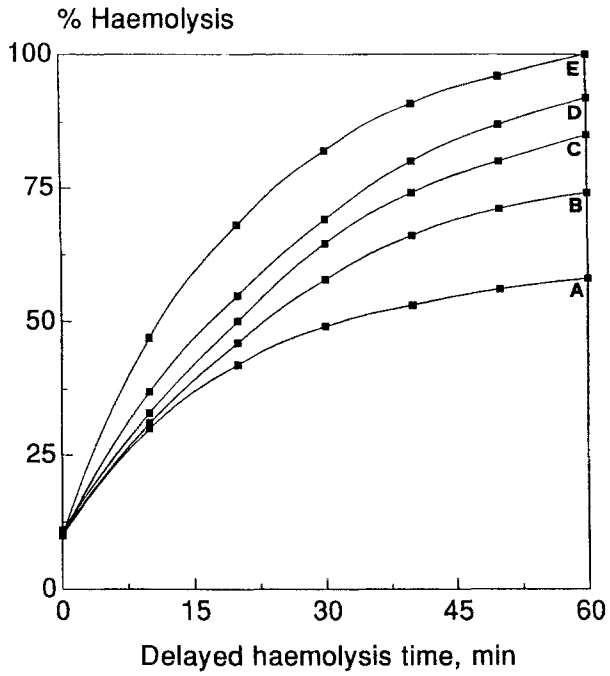

Fig. 9. Effect of β-Cyd added to RBC suspensions after the irradiation in the presence of NAP; [NAP] = 1.4×10^{-4} M; [RBC] = 3.3×10^{6} cells/mL; irradiation time 25 min; temperature 20°C. [β-Cyd]: A = 0; B = 0.7; C = 2.6; D = 3.5; E = 5.2×10^{-4} M. Each point is the mean ±SD of triplicate experiments.

with the increase of the irradiation time, as shown by the trend of the protection factors reported in the Y axis of Figure 10. This finding can demonstrate that the β-Cyd-induced modification of the membrane structure leading to its damage is favoured by the photosensitizing action of the drug. As a consequence the higher the photo-induced alteration, the lower the concentration of β-Cyd sufficient to further enhance the rate of photohaemolysis is.

3.3. Effect of β-Cyd on the photohaemolysis induced by Ketoprofen

Photohaemolysis induced by KPF is also known to be initiated, in addition to transient species as observed in the case of NAP, by stable photoproducts formed in the photodegradation reaction [2]. Also in this case, the results of the experiments of photohaemolysis carried out at various times of irradiation in the presence of KPF 4×10^{-5} M, and β-Cyd 8×10^{-5} M, confirm the reduction of the photohaemolytic efficiency of KPF in aerobic conditions due to the addition of β-Cyd, as shown in Figure 11.

Moreover, when a KPF 4×10^{-5} M solution was irradiated with constant RBC and variable β-Cyd concentration, the protective effect of cyclodextrin on the

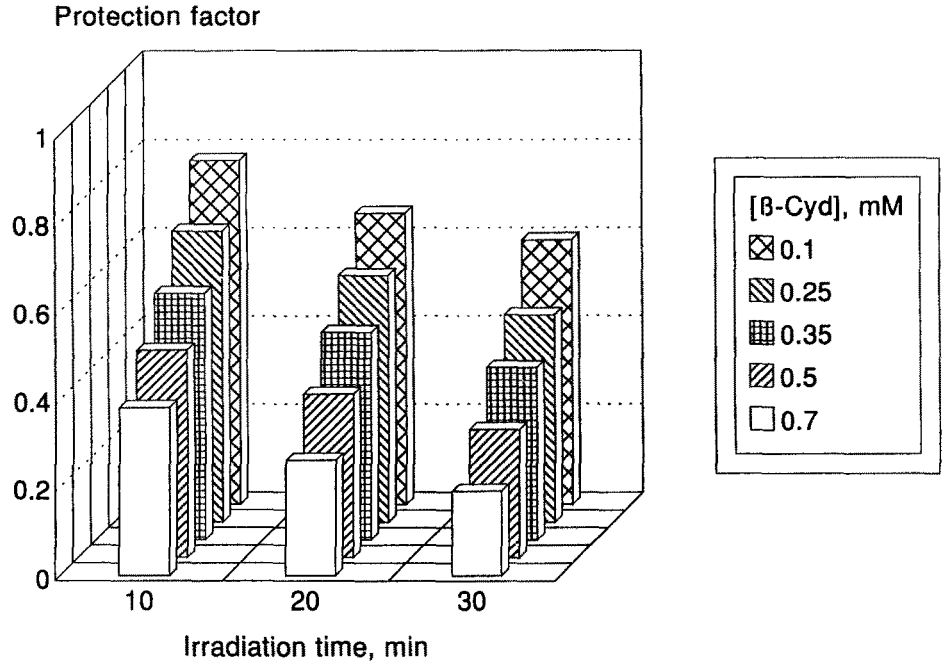

Fig. 10. Effect of β-Cyd added to RBC suspensions after various times of irradiation in the presence of NAP; [NAP] = 1.4×10^{-4} M; [RBC] = 3.3×10^{6} cells/mL; temperature 20°C. Each point is the mean ±SD of triplicate experiments.

photohaemolysis was found in a restricted range of concentration: $(0.2 - 2) \times 10^{-4}$ M. This protective effect was lower than NAP, considering the higher level of inclusion reached in the system KPF/β-Cyd (see Figure 12). These results can suggest the presence of a lytic agent, such as the photoproducts of KPF, which can not be influenced by the presence of β-Cyd.

In fact, the protective effect was hardly observed when RBC and cyclodextrin at variable concentrations were added to pre-irradiated solutions of KPF, in which a considerable amount of lytic photoproducts has been formed. In addition to these results, as observed in the experiments carried out with NAP as photosensitizer, the addition of β-Cyd to aliquots of irradiated RBC suspension in KPF solutions resulted in an increase of the rate of delayed haemolysis rate which is a function of the concentration of added β-Cyd and of the pre-irradiation time.

These results suggest that the protective action of β-Cyd as a complexing agent could be most efficient on the attack of the KPF radical, of the oxygen active species and of other transient species toxic towards the membrane, whereas the inclusion process, referred to the lytic photoproducts, did not seem to have a significant protective action, although at present it is not clear to what extent and in which manner the inclusion complexation participate in the KPF photoprotection.

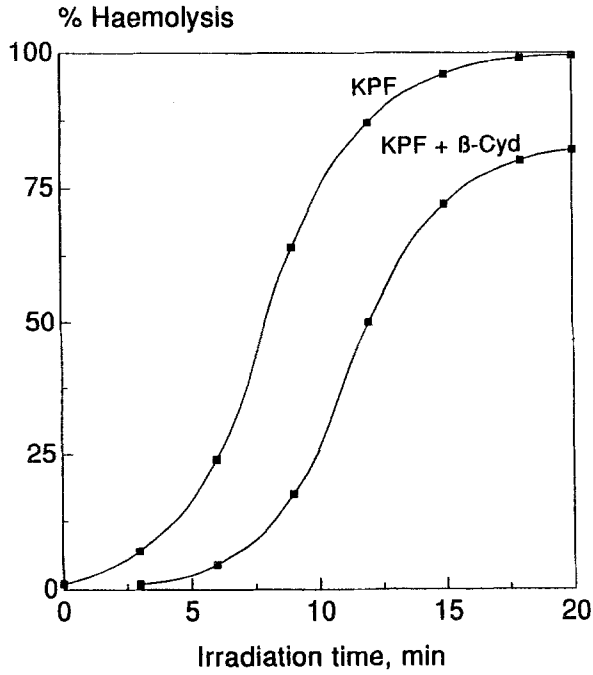

Fig. 11. Photohaemolysis of RBC sensitized by KPF in the presence or in the absence of β-Cyd. [KPF] = 4.2×10^{-5} M; [β-Cyd] = 8.0×10^{-5} M; [RBC] = 3.3×10^{6} cells/mL; temperature 20°C. Each point is the mean ±SD of triplicate experiments.

3.4. Effect of β-Cyd on the quantum yield of NAP and KPF photodegradation

The quantum yield Φ of the formation of the 6-MAN from the autophotooxidation of NAP in the presence of increasing amounts of β-Cyd was measured at 313 nm in aerobic conditions up to 10% conversion, when the absorption of the photoproduct was negligible, and thus the quantum yield can be calculated from the initial 6-MAN production rate, followed through the increase of the absorbance at 312 nm:

$$\frac{\mathrm{d[6\text{-}MAN]}}{\mathrm{d}t} = \Phi F I / v$$

where $F = 1 \times 10^{-A}$ is the fraction of light absorbed by CPF, I is the light intensity at the irradiation wavelength (mol of photons min^{-1}) and v is the solution volume.

The apparent quantum yield decreased with the increase of β-Cyd concentration, while it gave a constant result if the value of F was calculated by considering only the absorption of the light due to the uncomplexed NAP. The concentration of free sensitizer was calculated through the value of the stability constant.

Thus, the constancy of the value of Φ at 313 nm thus calculated ($1.2 \times 10^{-2} \pm 0.03$) suggests that the photooxidation of the starting compound *via* intermediate

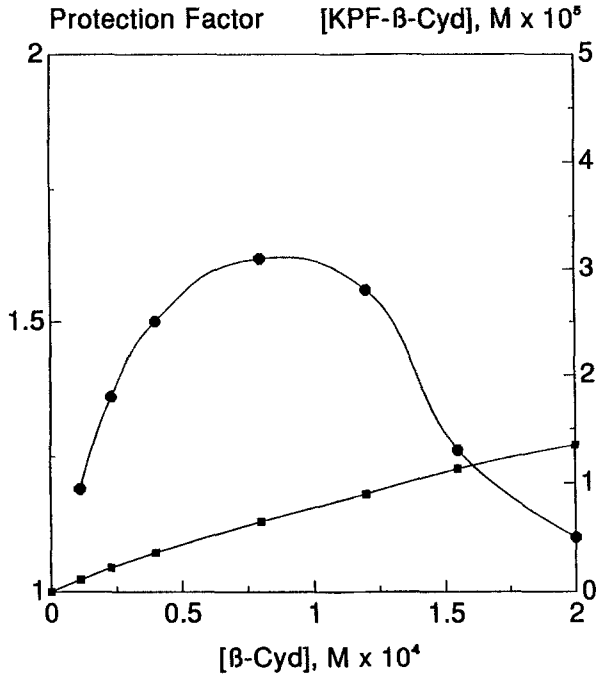

Fig. 12. Photohaemolysis of RBC sensitized by KPF in the presence of increasing amounts of β-Cyd. [KPF] = 4.2×10^{-5} M; [RBC] = 3.3×10^{6} cells/mL; irradiation time 15 min; (•) = Protection factors; (■) = [NAP–β-Cyd]; temperature 20°C. Each point is the mean ±SD of triplicate experiments.

radicals leading to the photoproduct 6-MAN occurs only for the uncomplexed molecule. This finding can justify the decrease in the photosensitizing properties of the molecule towards RBC in the presence of β-Cyd.

In the case of KPF the photodegradation of the starting compound leading to the photoproducts, described in the introductive section, was followed through HPLC of the irradiated solutions, which were extracted with cyclohexane–ethyl acetate (80 : 20 vol : vol) and chromatographed using the same eluant. Unfortunately, when the irradiation was performed in the presence of β-Cyd, the addition of the complexing agent led to the formation of a highly hydrophilic complex with the drug: thus the difficulties found in the extraction procedures did not permit us to obtain a quantitative evaluation of the quantum yield of KPF photodegradation.

3.5. Protective action of the complexes NAP–β-Cyd and KPF–β-Cyd

On the basis of the results reported above, the photosensitizing properties of the inclusion complexes of the two drugs with β-Cyd, prepared as described in the experimental section, were tested in the presence of red blood cells, to evaluate if their photohaemolytic activity is reduced compared with the uncomplexed molecules, and consequently if they can be used to some advantage in the therapeutical submministration.

TABLE I

Effect of the inclusion complexes NAP–β-Cyd and KPF–β-Cyd on the photohaemolysis of red blood cells in aerobic conditions.

Complex	Concentration (M)	Free drug (M)*	Protection factor#
NAP–β-CD	1.0×10^{-4}	0.9×10^{-4}	1.1 ± 0.04
	1.4×10^{-4}	1.2×10^{-4}	1.7 ± 0.07
	1.8×10^{-4}	1.5×10^{-4}	2.2 ± 0.11
	2.2×10^{-4}	1.7×10^{-4}	3.5 ± 0.18
	5.0×10^{-4}	3.3×10^{-4}	1.8 ± 0.10
	8.0×10^{-4}	4.7×10^{-4}	1.4 ± 0.05
	1.2×10^{-3}	6.0×10^{-4}	0.9 ± 0.01
KPF–β-CD	1.3×10^{-5}	1.2×10^{-5}	1.1 ± 0.06
	3.9×10^{-5}	3.5×10^{-5}	1.2 ± 0.06
	6.5×10^{-5}	5.6×10^{-5}	1.3 ± 0.05
	1.0×10^{-4}	7.6×10^{-5}	1.8 ± 0.09
	1.4×10^{-4}	1.1×10^{-4}	1.5 ± 0.07
	1.8×10^{-4}	1.3×10^{-4}	1.1 ± 0.04
	2.3×10^{-4}	1.6×10^{-4}	0.8 ± 0.05

Each value with ± designation is the mean ±SD of three experiments.

* Calculated through the K_s values of the complexes.

\# The protection factors are referred to a correspondent experiments carried out in the presence of uncomplexed drug in the same concentration of the complex.

Table I shows the results of the experiments of photohaemolysis at different concentrations of the complexes NAP–β-Cyd and KPF–β-Cyd, respectively; in this case, too, the interaction between β-Cyd and the cell membrane was neglected in the calculation of the free drug concentration. Each protection factor was referred to a corresponding experiment carried out in the presence of the uncomplexed drugs in the same concentration of the complex. The results confirm the validity of the inclusion process as a protective system towards a phototoxic side effect, but only in a restricted range of complex concentration ($0.1 - 1 \times 10^{-3}$ M and $0.1 - 2 \times 10^{-4}$ M for NAP–β-Cyd and KPF–β-Cyd, respectively). In fact, as observed previously, an increase in the complex concentration leads to levels of free β-Cyd which are toxic for the cell and thus contrast with the photoprotective action. This suggests the availability of this system in decreasing the adverse effects of drug photosensitization in biological systems, although it could be limited by the processes described above.

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