ELSEVIER International Journal of Pharmaceutics 162 (1998) 137–142

international journal of pharmaceutics

Drugs, in cyclodextrins, in liposomes: a novel approach to the chemical stability of drugs sensitive to hydrolysis

Yannis L. Loukas [1], Vassilia Vraka[1], Gregory Gregoriadis *

Centre for Drug Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK

Accepted 7 November 1997

Abstract

The entrapment in multilamellar liposomes of the inclusion complexes of cyclodextrins with drugs sensitive to hydrolysis, is being examined. The anti-inflammatory drug indomethacin (IND), rapidly hydrolyzed in alkaline media, can be stabilized 75-fold when its inclusion complex with the hydroxypropylated derivative of β-cyclodextrin (HPβCD) is entrapped in multilamellar liposomes. These liposomes were composed of egg phosphatidylcholine and cholesterol of 1:1 molar ratio and were prepared according to the dehydration-rehydration method. The molar ratio for the IND:HPβCD inclusion complex was estimated to be 1:1 using the continuous variation method based on the ^{1}H NMR data. Furthermore, the binding constant (K_{11}) of the IND:HPβCD complex was estimated kinetically and by ^{1}H NMR. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cyclodextrins; Indomethacin; β-Cyclodextrin; Egg phosphatidylcholine

---

1. Introduction

In aqueous solutions almost all of the drugs are subject to some form of chemical degradation and, frequently, the therapeutic activity is impaired by the drug instability (Loftsson and Brewster, 1996). The most common consequence of the drug's degradation is the loss of potency but in some cases harmful degradation products may be formed. For example, it is well known that allergy is, in many cases, due to drug degradation products rather than to the drug itself. Various methods have been used to increase to some extent drug stability in aqueous solvent systems such as adjustments of pH of the aqueous solution, the presence of drugs in buffered liposomal suspensions, the complexation of drugs with various cyclodextrins and the formation of gels or emulsions (Carstensen, 1990). However, there is a need

---

* Corresponding author. Tel.: +44 171 7535822; fax: +44 171 7535820.

[1] Present address: Riga Ferreou 21, Ano Ilioupolis, 163 43 Athens, Greece.

PII S0378-5173(97)00421-3

to increase further the stability against hydrolysis, especially in therapeutics where a wide variety of drugs is administered in unstable forms.

A new approach, based on the entrapment of cyclodextrin inclusion complexes of labile drugs in multilamellar liposomes, greatly increases their chemical stability (Gregoriadis and Loukas, 1994). It has been recently proposed that drugs sensitive to light and photochemical oxidation, such as riboflavin (Loukas et al., 1995a,b) and sodium ascorbate (Loukas et al., 1996a) respectively, exhibited a great increase in their stability (270- and 125-fold, respectively) when entrapped as cyclodextrin complexes in multilamellar liposomes incorporating light absorbers (such as oil red O, oxybenzone, deoxybenzone, sulisobenzone) and antioxidants (such as β-carotene) in their bilayers.

In the present study, the stability of drugs sensitive to hydrolysis is being examined. The stability of the anti-inflammatory drug indomethacin, which is hydrolyzable in alkaline buffered solutions, was increased 75-fold when entrapped in multilamellar liposomes in the form of a hydroxypropyl-β-cyclodextrin 1:1 inclusion complex. Previous efforts from the literature (Loftsson and Brewster, 1996) to stabilise indomethacin have ended in a 6-fold increase in its stability.

2. Experimental section

2.1. Materials and instrumentation

Indomethacin (IND) was purchased from Aldrich (Dorset, UK) and HPβCD from Janssen (Beerse, Belgium). IND (free acid form), converted into sodium salt by titration with NAOH in H_2O to pH 7.8 and the solution was then freeze–dried. Double-distilled water was used throughout. All other reagents were of analytical grade. The kinetics of IND hydrolysis in buffer solutions (pH 9, at 30°C), were performed and monitored spectrophotometrically (at 321.4 nm) in a Compuspec UV/visible spectrophotometer (Wallac) connected to a personal computer. Calculation of the IND:HPβCD binding constant in aqueous solution was carried out by ^{1}H-NMR spectroscopy. ^{1}H-NMR spectra were obtained in NaOD (pH 9, at 30°C) recorded on a Brucker AM 500 spectrometer connected to an Aspect 3000 computer. The chemical shifts were related to the residual solvent signal (hydrogen-deuterium chloride = 4.84 ppm at 303 K). Typical conditions were 16 K data points with zero filling, sweep width of 5 KHz giving a digital resolution of 0.61 Hz point^{-1}, pulse width 4 μs, acquisition time 1.64 s and number of scannings 16.

2.2. Preparation methods

The inclusion complex of IND with the HPβCD derivative was prepared by the freeze-drying method (Loukas et al., 1997). HPβCD (0.08 mmol) were dissolved in 5 ml distilled water and the clear solution was added dropwise in an aqueous solution of IND (0.08 mmol in 5 ml) under continuous stirring. The mixtures were allowed to stir in the absence of light at room temperature and then filtered through a 0.22 μm membrane filter unit (Millex-HV, Millipore, Bedford, MA). The filtrates were freeze–dried to yield amorphous powder.

Entrapment of IND in free or complexed form, into liposomes was carried out by the dehydration-rehydration method (Kirby and Gregoriadis, 1984). Small unilamellar vesicles (SUV) prepared at the appropriate temperatures from PC (19 mmol) and equimolar cholesterol were mixed with 1.9 mmol of free or complexed IND dissolved in water and freeze–dried overnight. The dry powder was subjected to controlled rehydration at the same temperatures, initially with water and finally with 0.1 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS) and centrifuged at 27 300 g for 20 min to separate the entrapped from non-entrapped IND. The liposomal pellet containing multilamellar dehydration-rehydration vesicles (DRV) was washed thrice in PBS and resuspended in 4 ml PBS until further use.

Table 1
Entrapment values, hydrolysis rates and stabilization ratios of IND in DRV formulations exposed to pH 9

Sample	Entrapment values (%)	k ($\times 10^4$ min^{-1})	Stabilization ratio
IND	—	462	1
IND:HPβCD	—	132	3.5
IND in DRV[a]	41	21	22
IND:HPβCD in DRV[a]	24	6	75
IND:HPβCD in DRV[b]	15	15	31

[a]Composed of eggPC:cholesterol 1:1.
[b]Composed from eggPC:cholesterol 1:0.5.

2.3. Continuous variation method

A reliable determination of the IND:HPβCD stoichiometry can be provided by the continuous variation technique (Job plot, Connors, 1987), based on the difference in chemical shift $\Delta\delta$ ($\Delta\delta = \delta_0 - \delta$) of IND observed in the presence of HPβCD. Equimolar solutions of IND and HPβCD were prepared and mixed to standard volume and proportions in order for the total concentration to remain constant ($[\text{IND}]_t + [\text{HP}\beta\text{CD}]_t = M$). $\Delta\delta$ values in the preparations of IND were calculated by measuring the chemical shift of IND in the absence (δ_0) and presence (δ) of HPβCD. Subsequently, $\Delta\delta[\text{IND}]_t$ was plotted against r ($r = ([\text{IND}]_t)/([\text{IND}]_t + [\text{HP}\beta\text{CD}]_t)$). Generally, the concentrations of the free guest (G) and the cyclodextrin (CD) in a 1:n inclusion complex (G:CD$_n$) can be expressed as follows:

$$[G] = rM - [G{:}\text{CD}]$$

$$[\text{CD}] = M(1-r) - n[G{:}\text{CD}]$$

For a given value of r, the concentration of the complex G:CD will reach a maximum corresponding to the point where the derivative $\text{d}[G{:}\text{CD}]/\text{d}r = 0$. Derivation of the above two equations according to r give the following: $\text{d}[G]/\text{d}r = M$ and $\text{d}[\text{CD}]/\text{d}r = -M$. Rearrangement of the above equations leads to a single solution: the maximal absolute complex concentration is reached for $r = (n+1)^{-1}$ and does not depend on M or on the binding constant.

2.4. Kinetic studies

The kinetics of IND hydrolysis in free, complexed and entrapped in liposomes forms, were monitored spectrophotometrically. Buffered solutions with constant concentration of IND either in free form or in different formulations (Table 1) were stirring in order to be homogeneous. At time intervals, a standard proportion was withdrawn, diluted to a standard volume and the decrease in absorbance at 321.4 nm was measured spectrophotometrically. In the case of the formed liposomes, the pellets were disrupted first using isopropanol. Furthermore, the binding constant for IND:HPβCD complex was calculated kinetically in order to evaluate the values resulting from the NMR studies. To this end, the hydrolysis kinetics were performed in IND buffered solutions containing increasing concentrations of HPβCD.

3. Results and discussion

3.1. Determination of IND entrapped in DRV liposomes

The isolated, from the preparation procedure, pellets were first dissolved with isopropanol and IND entrapment values were calculated by UV spectrophotometry. Furthermore, the pooled supernatants were measured for the unentrapped

IND by disrupting possible small unilamellar vesicles (SUV) with isopropanol. The three combined supernatants were also measured by UV spectrophotometry. IND entrapment values (Table 1) were calculated according to the formula:

$$\%\,\text{entrapment} = \frac{A_P}{A_P + A_S}\,100$$

where A_P is the absorbance of IND in the pellets and A_S is the absorbance of non-entrapped IND in the pooled supernatants, after a dilution correction to achieve identical dilutions for both A_P and A_S.

3.2. Determination of the complex stoichiometry

To determine the complex stoichiometry using the continuous variation method, NMR spectra were obtained for IND and HPβCD mixtures, in which the total initial concentrations of the two species were maintained constant but the ratio of initial concentrations r (Section 2) varied between 0 and 1. If a physical parameter directly related to the concentration of the complex (for instance the chemical shift δ) can be measured under these conditions, and is then plotted as a function of r, the maximal value for this parameter will occur at $r = m/m + n$, where m and n are IND and HPβCD proportions in the complex, respectively (IND$_m$:HPβCD$_n$). This means that if the complex stoichiometry is 1:1 (m, $n = 1$), the maximum value for the examined parameter will be reached at $r = 0.5$; (if the complex stoichiometry is 1:2 the maximum value will be reached at $r = 0.33$). The calculated quantities $\Delta\delta[\text{IND}]_t$ are proportional to the concentration of the complex, and can be thus plotted against r. The resulting continuous variation plot demonstrate that since the maximum has an r value of almost 0.5 for IND:HPβCD (Fig. 1), the complex has a 1:1 stoichiometry.

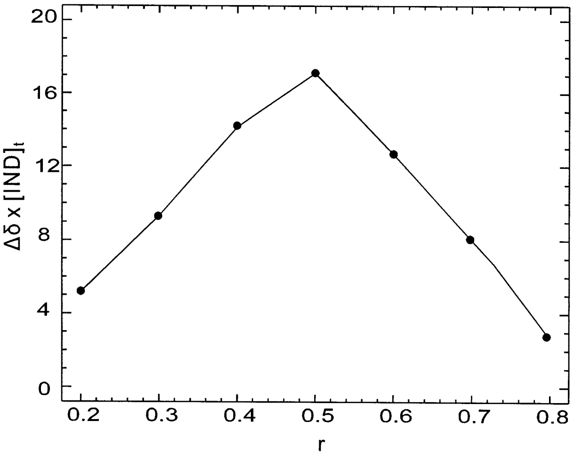
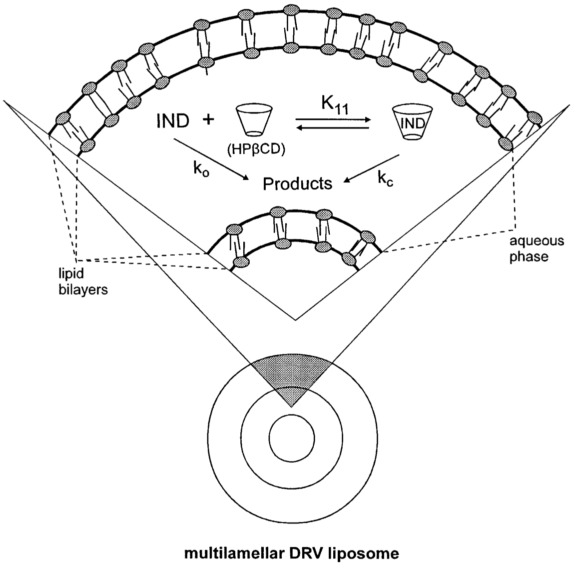


Fig. 1. Continuous variation plot (Job plot) of IND:HPβCD.

3.3. Hydrolysis studies

The hydrolysis of the entrapped IND in free or complexed form is presented schematically in Scheme 1. In the case of free IND, only the rate constant k_0 is measured during the hydrolysis studies. In the case of the 1:1 IND:HPβCD inclusion complex, a two-way reaction mechanism (Scheme 1) is taking place, where k_0 is the hydrolysis rate constant for the free IND, k_c the hydrolysis rate constant for the complexed IND and K_{11} is the binding constant for the formed complex. Since the binding constant (K_{11}) gives an idea of how 'stable' an inclusion complex is and plays a



Scheme 1. Schematic representation of a DRV multilamellar liposome, showing the degradation pathways inside the aqueous phase. For simplification, the cholesterol molecules are omitted.

substantial role in interpreting the chemical stability of the included drug, it must be calculated and evaluated using different methods; for instance low values for K_{11} (<100 M^{-1}) give the idea of a 'weak' complex, whereas high values (>500 M^{-1}) give the idea of a 'stable' complex. Linear models (Loukas et al., 1995c, 1994) and a recently described (Loukas et al., 1996b) nonlinear one (Eq. (1)), illustrate the kinetic behaviour of the guest in the presence of CDs and are usually solved using either linear regression or specific algorithms, such as the Marquardt iterative algorithm.

$$\Delta k_{\mathrm{obs}} = K_{11}\left(1 - \frac{\Delta k_{\mathrm{obs}}}{\Delta k_{\mathrm{c}}}\right) \times \left([\mathrm{HP}\beta\mathrm{CD}]_{\mathrm{t}} - \frac{[\mathrm{IND}]_{\mathrm{t}}\Delta k_{\mathrm{obs}}}{\Delta k_{\mathrm{c}}}\right)\Delta k_{\mathrm{c}} \qquad (1)$$

where

$$\Delta k_{\mathrm{obs}} = k_0 - k_{\mathrm{obs}}$$

and

$$\Delta k_{\mathrm{c}} = k_0 = k_{\mathrm{c}}$$

.

Eq. (1) correlates the initial total concentrations $[\mathrm{IND}]_{\mathrm{t}}]$ and $[\mathrm{HP}\beta\mathrm{CD}]_{\mathrm{t}}$ with the hydrolysis rate constant k_0 of free IND and the observed rate constant k_{obs} in the presence of HPβCD the unknown parameter K_{11} (950 M^{-1}) can be calculated according to this model.

3.4. Evaluation of the binding constants using NMR

The binding constant K_{11} for the examined 1:1 complex (IND:HPβCD) was determined also by ^{1}H NMR spectroscopy from the NMR spectra of IND in the presence of increasing concentrations of HPβCD (Fig. 2). During the complexation procedure of IND with HPβCD, if only shifts of the spectral lines are observed, due to fast averaging by the exchange between free and complexed states, then, for a signal belonging to IND, the quantity $\Delta\delta[\mathrm{IND}]_{\mathrm{t}}$ will be proportional to the complex concentration [IND:HPβCD]. Correlating the $\Delta\delta$ of a specific proton of IND (H6, Fig. 2) in the presence of HPβCD, using the Benesi-Hildebrand graphical method, (Eq. (2)), the K_{11} (715 M^{-1}) was calculated from the slope $(K_{11}\Delta\delta_{\mathrm{c}})^{-1}$ and intercept $(\Delta\delta_{\mathrm{c}})^{-1}$ of the resulting line (not shown).

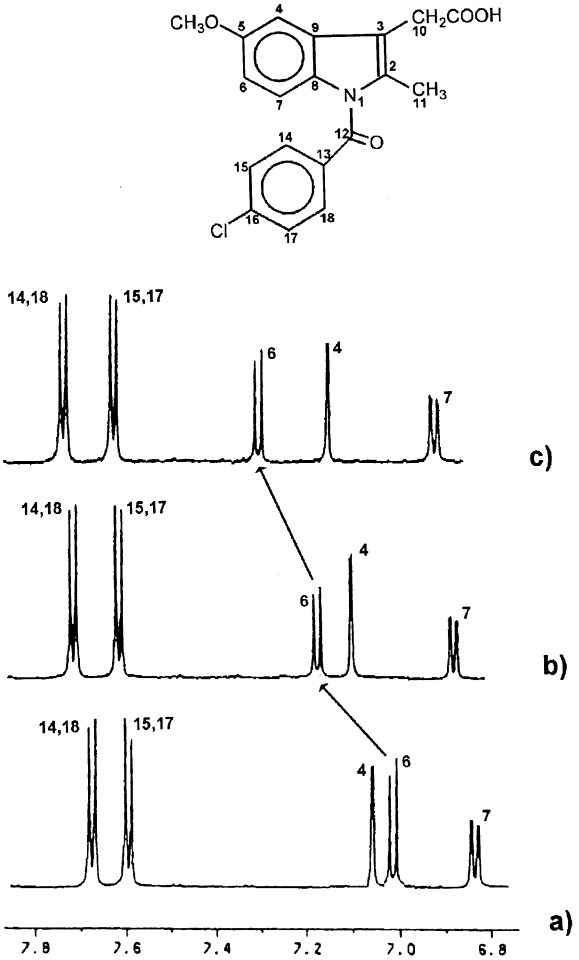


Fig. 2. Partial 500 MHz ^{1}H NMR spectra of IND:HPβCD mixtures: (a) 10 mM IND; (b) 7 mM IND and 3 mM βCD; and (c) 5 mM IND and 5 mM βCD.

$$\frac{1}{\Delta\delta} = \frac{1}{K_{11}\Delta\delta_{\mathrm{c}}}\frac{1}{[\mathrm{HP}\beta\mathrm{CD}]_{\mathrm{t}}} + \frac{1}{\Delta\delta_{\mathrm{c}}} \qquad (2)$$

$\Delta\delta_{\mathrm{c}} = \delta_0 - \delta_{\mathrm{c}}$, where δ_0 and δ_{c} are, for a given proton, the chemical shifts of IND in free and complexed form.

From the calculated values of K_{11}, using both methods and the stability studies (Table 1) it can be concluded that IND:HPβCD is a 'stable' complex and remains mostly in the form of a complex inside the aqueous phase of the DRVs, since there is no competition for the hydrophobic cavity between the six-membered ring of the indole unit of IND (Fig. 2) and the lipids polar heads (Scheme 1). Therefore, the increased size of the complexed IND could be the main reason for the great increase of its stability. The leaking rate of the bulky complex from the DRVs is considerably slower comparing to the leaking rate of the free drug (Loukas and Gregoriadis, 1997). Furthermore, since the aqueous phases of DRVs are almost neutral in pH (see above), the drug could remain stable inside the DRVs. Hence, from the above, it results that the higher the retention of the drug in the DRVs the higher the stability. Finally, from the data in Table 1 it is becoming evident that the 1:1 molar ratio between egg PC and cholesterol, produces the highest physical stability for the DRVs, comparing to DRVs with 1:0.5 egg PC:cholesterol molar ratio, as we have concluded elsewhere. The role of cholesterol in the present case could be to reduce the fluidity of the lipids membrane reducing substantially the leakage of the entrapped materials. The described system can present three lines of defense against hydrolysis, through the presence of the lipid bilayers in a hydrophobic assembly, the hydrophobic cavity of the cyclodextrin harboring the unstable compound and the considerable decrease in IND:HPβCD leaking rate, probably due to the enhanced size of the complex or to the possible interaction (hydrogen bonds) between the lipids polar heads and the external part of cyclodextrins.

In conclusion, the novel combination of these two supramolecular systems (liposomes and cyclodextrins), dynamically increases the moderate stabilizing effect of each one separately. Since liposomes remain almost stable in the pH range of the biological milieu, the described system could play the 'microreservoir' role for sensitive to hydrolysis substances.

References

Carstensen, J., 1990. Drug Stability: Principles and Practices. Marcel Dekker, New York.

Connors, K.A., 1987. Binding Constants, the Measurement of Molecular Complex Stability. Wiley, New York.

Gregoriadis, G., Loukas, Y.L., 1994. British patent, GB94.1111.50.

Kirby, C., Gregoriadis, G., 1984. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. Biotechnology 2, 979–984.

Loftsson, T., Brewster, M.E., 1996. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J. Pharm. Sci. 85, 1017–1025.

Loukas, Y.L., Gregoriadis, G., 1997. Drugs, in cyclodextrins, in liposomes: a novel approach for the reduction of drug leakage from liposomes. Proceedings of the Twentyfourth International Symposium on Controlled Release of Bioactive Materials. Stockholm, Sweden, June 15–19.

Loukas, Y.L., Vyza, A.E., Valiraki, A.P., 1994. β-Cyclodextrin inclusion complex of a new organophosphorus insecticide. Determination of stability constant with HPLC. J. Agric. Food Chem. 42, 944–948.

Loukas, Y.L., Jayasekera, P., Gregoriadis, G., 1995a. Novel liposome-based multicomponent systems for the protection of photolabile agents. Int. J. Pharm. 117, 85–94.

Loukas, Y.L., Jayasekera, P., Gregoriadis, G., 1995b. Characterization and photoprotection studies of a model β-cyclodextrin-included photolabile drug entrapped in liposomes incorporating light absorbers. J. Phys. Chem. 99, 11035–11040.

Loukas, Y.L., Vyza, A.E., Valiraki, A.P., 1995c. Inclusion complexes and stability studies of an organophosphorus insecticide with cyclodextrins: Spectrophotometric and kinetic determination of stability constant. Analyst 120, 533–538.

Loukas, Y.L., Vraka, V., Gregoriadis, G., 1996a. Entrapment of sodium ascorbate-β-cyclodextrin inclusion complex in multilamellar liposomes containing light absorbers, greatly increases the stability of the vitamin against photochemical oxidation. Pharm. Sci. 2, 523–527.

Loukas, Y.L., Vraka, V., Gregoriadis, G., 1996b. Use of a nonlinear least-squares model for the kinetic determination of the stability constant of cyclodextrin inclusion complexes. Int. J. Pharm. 144, 225–231.

Loukas, Y.L., Vraka, V., Gregoriadis, G., 1997. New nonacidic formulations of haloperidol complexed with β-cyclodextrin derivatives. J. Pharm. Biomed. Anal. 16, 263–268.