

## Original article

Physico-chemical characterization of disoxaril–dimethyl- $\beta$ -cyclodextrin inclusion complex and in vitro permeation studiesCinzia Anna Ventura<sup>a,\*</sup>, Ignazio Giannone<sup>b</sup>, Teresa Musumeci<sup>b</sup>, Rosario Pignatello<sup>b</sup>,  
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## Abstract

In this work we evaluated the ability of 2,6-di-*O*-methyl- $\beta$ -cyclodextrin (DM- $\beta$ -Cyd) to include the anti-rhinovirus drug Disoxaril (WIN 51711), increasing its water solubility and stability. The complex, prepared by kneading method, was characterized in the solid state by differential scanning calorimetry and in aqueous solution using circular dichroism and NMR spectroscopy. The formation of 1:1 and 1:2 drug–Cyd complexes was hypothesized. Stability constants for both complexes were determined on the basis of an Ap-type phase solubility diagrams and evidenced a very high stability for the 1:1 complex. Thermodynamic parameters of the binding process showed the existence of classical hydrophobic interactions in the 1:1 complex with the formation of a less ordered system after complexation. An enthalpic contribution rather than an entropic one accompanied the association of the second Cyd molecule. DM- $\beta$ -Cyd was able to significantly increase water solubility of WIN 51711, from 0.000123 to 0.47142 mg/ml. Free drug shows a very low water stability, it is completely hydrolyzed after 36 h in PBS (pH 7.0), at 4 °C. In the presence of DM- $\beta$ -Cyd only a 10% of WIN 51711 was degraded, to the same conditions, after 12 days. DM- $\beta$ -Cyd increases the permeation of WIN 51711 across excised bovine nasal mucosa mounted on Franz cells, with respect to the free drug. Nevertheless, the permeation process had a lag time of 2 h so that the drug might assure its pharmacological activity on the outer surface of the mucosa. In vivo studies on rabbits evidenced that WIN 51711 is well tolerated, having no observable effect on the nasal mucosa following repeated administration.

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**Keywords:** Disoxaril; Dimethyl- $\beta$ -cyclodextrin; Inclusion complex; Physico-chemical characterization; Permeation; Nasal mucosa

## 1. Introduction

The rhinoviruses (RVs) are responsible for more than half of all cases of the common cold. Infections by RVs are associated with a number of upper and lower respiratory tract complications in both adults and children [1]. A variety of antiviral agents that inhibit viral uncoating have been reported for the rhino- and enterovirus members of the picornavirus family [2]. A new series of compounds developed by Sterling–Winthrop Pharmaceuticals and namely WIN has been clinically evaluated

in RVs infections. The first compound of this group to advance to clinical trials was Disoxaril (WIN 51711) [1]. WIN 51711 inhibits picornavirus replication in tissue culture and in animal models of human enterovirus disease. Its action is due to a binding with the hydrophobic interior of viral capsid, causing significant conformational changes in enlargement of the pocket within the capsid. In this way, WIN 51711 stabilizes the capsid by making the coat proteins sufficiently rigid to prevent their normal assembly and disassembly functions [2]. Although WIN 51711 is a very active anti-Rhinovirus agent, it was withdrawn from clinical studies due to the appearance of asymptomatic crystalluria probably due to the high used dosage levels in healthy volunteers and the low concentration found after oral administration in the entrance site of RVs represented by nasal

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mucosa [3]. To avoid this side effect and increase the concentration on nasal mucosa a topical administration of the drug could be useful.

WIN 51711 is a 5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methyl-isoxazole (Fig. 1) and due to its hydrophobic properties, it is characterized by a very low water solubility that does not allow to prepare liquid formulations. WIN 51711 is quite soluble at acidic pH due to the salt formation with the oxazoline nitrogen; however, these conditions produce a rapid degradation of the drug. The hydrolysis products were identified and found to have little or no antiviral activity due to the disappearance of the oxazole moiety and the appearance of the aminoester and the amide moieties [4]. To improve the biopharmaceutical properties of this drug, it could be necessary to increase its water solubility, maintaining its chemical structure.

In recent years, to overcome the stability and solubility problems of drugs, several approaches have been investigated. Cyclodextrins (Cyds) have been extensively used as complexing agents to improve solubility and stability of a variety of poorly soluble and labile drugs. Natural Cyds have limited water solubility that negatively influences water solubility of the formed complex. To overcome this problem alkyl moiety such as hydroxyalkyl or methyl on free hydroxyl groups of  $\beta$ -Cyd were introduced. The complexing ability of Cyd derivatives was significantly modified in respect to the parents, for example 2,6-di-*O*-methyl- $\beta$ -cyclodextrin (DM- $\beta$ -Cyd) shows higher affinity for various drugs and then higher solubilizing ability compared to natural  $\beta$ -Cyd [5].

DM- $\beta$ -Cyd has shown to be a useful excipient in nasal drug delivery. After a single nasal administration of 2% DM- $\beta$ -Cyd, its acute histological effects on the epithelium of the nasal cavity were similar to those of saline [6]. No side effect on the mucociliary clearance was found [7]. In humans, DM- $\beta$ -Cyd was scarcely absorbed after nasal administration. The application of a spray containing 2% and 5% of DM- $\beta$ -Cyd revealed not more than 4% of the administered dose in urine [8].

In this work we investigated the ability of DM- $\beta$ -Cyd to include WIN 51711, influencing its solubility and stability in water solution. The complex was prepared by kneading method using different DM- $\beta$ -Cyd molar ratios and was investigated in the solid state by differential scanning calorimetry (DSC). Circular dichroism (CD), UV and  $^1\text{H}$ -NMR spectroscopy were used to obtain information about the existence of the complex in solution. Stability constant of the complex ( $K$ ) was determined by Higuchi and Connors' method (1965) [9] in phosphate buffered solution (pH 7.0) at different temperatures (5, 15 and 25 °C). Furthermore, the influence of DM- $\beta$ -Cyd on stability of WIN 51711 was investigated. In vitro studies through excised bovine nasal mucosa were performed to eval-

uate the role of the macrocycle on nasal administration of the drug. In vivo studies were performed to assess tolerance to WIN 51711 when administered by the intranasal route to the rabbit over a period of 14 days.

## 2. Results and discussion

Solid samples obtained by the kneading method were analyzed in the solid state by DSC analysis. The obtained thermograms for the complexes compared to pure components and the physical mixture are showed in Fig. 2.

In the DSC curve of WIN 51711–DM- $\beta$ -Cyd 1:2 kneaded sample (curve E) the disappearance of the fusion peak at 100 °C relative to the pure drug is evident. This peak is instead present in the thermogram of the physical mixture. These findings demonstrated the presence of an interaction between the drug and Cyd in the solid state. Moreover, the exothermal peak observed at 130 °C in the thermogram of the solid sample could be due to the passage from an amorphous to a crystalline, due to heating [10]. The presence of drug fusion peak in the 1:1 kneaded sample (curve D) could evidence a sub-total interaction.

More information about the existence of an inclusion complex between WIN 51711 and DM- $\beta$ -Cyd could be obtained by solution studies. UV, CD and  $^1\text{H}$ -NMR spectroscopy were performed to evaluate the influence of Cyd on spectral characteristics of the drug. Fig. 3 shows UV spectra of free WIN 51711 and in the presence of different concentrations of DM- $\beta$ -Cyd. The drug shows an absorption band at 260 nm due to the  $\pi \rightarrow \pi^*$  transition of the phenylic moiety and a second band at 211 nm, probably due to  $n \rightarrow \pi^*$  transition of isoxazole nucleus [11]. To verify the attribution of the band at 211 nm, the drug was hydrolyzed in an acidic medium (HCl 0.1 N) at 50 °C for 12 h and the resulting aminoester (Fig. 4) [4] was freeze-dried

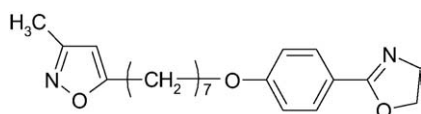


Fig. 1. Chemical structure of WIN 51711.

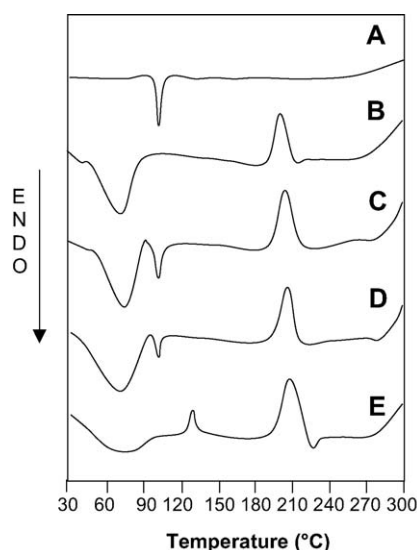


Fig. 2. DSC thermograms of WIN 51711–Cyd solid systems. A) WIN 51711 alone; B) DM- $\beta$ -Cyd alone; C) WIN 51711–DM- $\beta$ -Cyd 1:2 physical mixture; D) WIN 51711–DM- $\beta$ -Cyd 1:1 solid sample; E) WIN 51711–DM- $\beta$ -Cyd 1:2 solid sample.

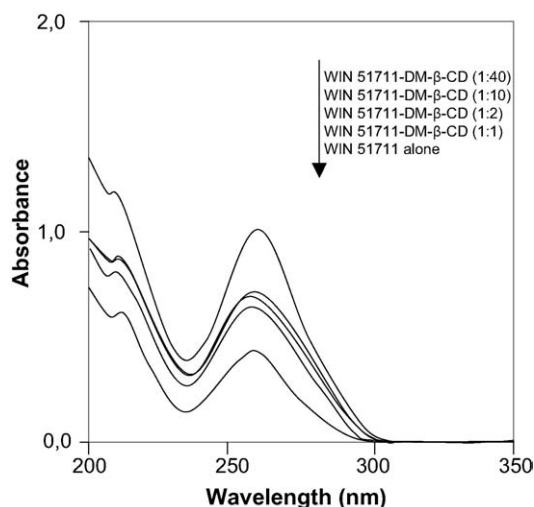


Fig. 3. UV spectra of WIN 51711 alone or in the presence of DM-β-Cyd in different molar ratio.

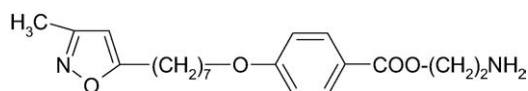


Fig. 4. Chemical structure of degraded WIN 51711.

(EDWARDS, Modulyo, 4 K). The obtained solid sample was solubilized in PBS (pH 7.0)/methanol solution (50:50, v/v) and submitted to UV spectroscopy. No variation was observed for the aminoester spectrum with respect to intact WIN 51711 (spectra not showed), thus evidencing that the absence of oxazole nucleus produces no variation in the UV absorption band of the drug.

DM-β-Cyd is able to influence both absorption bands of WIN 51711 at all the considered concentrations, producing a hyperchromic effect. The influence was of course more significant at the highest DM-β-Cyd concentration (1:40 drug/Cyd molar ratio). The observed trend was due to a perturbation of the chromophore electrons of the drug due to the inclusion into the Cyd cavity; because the phenyl and isoxazole nucleus represent the extreme opposite groups of WIN 51711 molecule, we hypothesized that two DM-β-Cyd molecules were involved for each drug molecule. It is probably that the drug deeply penetrated with its oxazole moiety within DM-β-Cyd cavity, so as to permit the partial inclusion of the phenyl group. This molecular structure could be confirmed by the spectra performed on hydrolyzed WIN 51711 in the presence of different concentrations of DM-β-Cyd. In this new chemical structure oxazole group is absent. Obtained UV spectra were practically overlapped to those of not hydrolyzed WIN 51711 (spectra not shown).

On the same solutions used for UV analysis, CD spectroscopy studies were performed. The obtained spectra are showed in Fig. 5. Free WIN 51711 showed no CD band, but in the presence of high DM-β-Cyd concentration two induct CDI bands were observed, one negative at 211 nm and the second positive at 260 nm, corresponding to UV absorption bands. The presence of these bands evidenced a perturbation

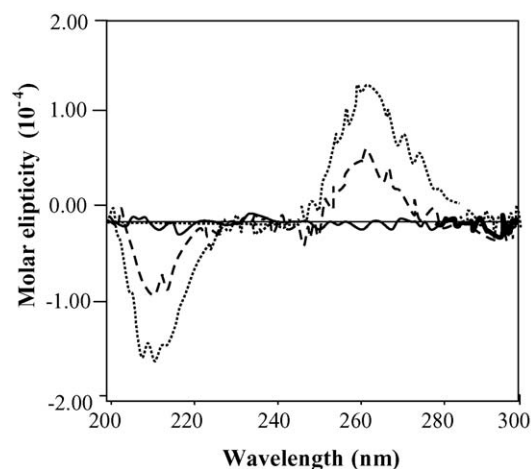


Fig. 5. CD spectra of WIN 51711 alone (—); WIN 51711 in the presence of DM-β-Cyd (1:10 molar ratio) (---); WIN 51711 in the presence of DM-β-Cyd (1:40 molar ratio) (·····).

of microenvironment polarity of WIN 51711 as a consequence of the inclusion into the DM-β-Cyd cavity. Harata and Uedaira (1975) [12] reported that the appearance of CD bands is due to spatial disposition of the electronic chromophore into the Cyd cavity. A positive band is related to parallel orientation of the dipole moment of the drug with respect to the Z-axis of the macrocycle cavity. Conversely, a negative band evidenced a perpendicular orientation of the dipole moment. On this basis, it can be hypothesized that the WIN 51711 phenyl group was deeply included in the macrocycle assuming an axial orientation. The isoxazole nucleus was probably superficially included in a second Cyd molecule assuming an equatorial orientation. Similar results were obtained for hydrolyzed WIN 51711 (data not showed).

The formulated hypotheses were confirmed by <sup>1</sup>H-NMR spectroscopy. In Table 1 we report the shifts observed for WIN 51711 in the presence of DM-β-Cyd (1:2 molar ratio).

Table 1  
<sup>1</sup>H-chemical shifts of WIN 51711 alone and in the presence of DM-β-Cyd in 1:2 molar ratio

| Protons | WIN 51711 alone | Drug-DM-β-Cyd inclusion complex | Δδ <sup>a</sup> |
|---------|-----------------|---------------------------------|-----------------|
| H4      | 4.001           | 4.011                           | 0.010           |
| H5      | 4.482           | 4.498                           | 0.016           |
| H7–11   | 7.822           | 7.848                           | 0.026           |
| H8–10   | 6.988           | 6.974                           | −0.014          |
| H13     | 4.063           | 4.068                           | 0.005           |
| H14     | 1.803–1.733     | —                               | —               |
| H15–18  | 1.520–1.495     | —                               | —               |
| H16–17  | —               | —                               | —               |
| H19     | 2.755           | 2.775                           | 0.020           |
| H24     | 6.045           | 6.060                           | 0.015           |
| H25     | 2.223           | 2.245                           | 0.022           |

<sup>a</sup> Δδ = δ<sub>complex</sub> − δ<sub>free</sub>.

All drug protons were influenced by the macrocycle. The downfield shifts observed for phenyl H7–11, H4 and H5 (oxazole group) of the drug could be attributed to a change in the local polarity [13] or a deshielding effect due to van der Waals forces between the aromatic moiety and carbohydrate chains [14], as a result of complexation within DM- $\beta$ -Cyd cavity. The upfield shifts observed for H8–10 protons could be due to a shielding effect produced by oxygen atoms of methoxyl group of the macrocycle that were in the proximity of the drug. This trend can confirm the results obtained by UV and CD spectroscopy. In fact, it could be hypothesized that DM- $\beta$ -Cyd included deeply the oxazoline moiety and partially the phenyl ring, so that protons in ortho to the oxazoline moiety were allocated into the cavity (downfield shifts), instead meta protons were in the proximity of methoxyl group of DM- $\beta$ -Cyd. As concerns the isoxazole nucleus, both H24 and methyl protons (H25) were downfield shifted confirming the inclusion of this group into DM- $\beta$ -Cyd cavity.

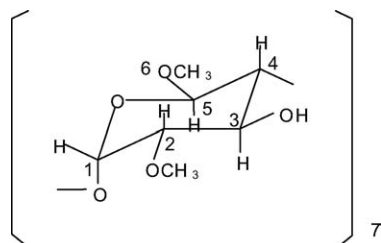
DM- $\beta$ -Cyd showed a significant upfield shift for H-3 and H-5 protons in the presence of the drug (Table 2). This trend was due to a shielding effect produced by the anisotropic current of WIN 51711 aromatic group that was included into the Cyd cavity and suggests that the drug penetrates deeper into the Cyd cavity. The shifts of H-1 and H-4 protons could be due to a change in the macrocycle rigidity due to the complexation.

The influence of DM- $\beta$ -Cyd on water solubility of WIN 51711 was evaluated in PBS at pH 7.0. The drug shows the less water solubility and the highest stability at this pH value. The solubility phase isotherms obtained using the Higuchi and Connors' method (1965) [9], at different temperatures (5, 15 and 25 °C) were showed in Fig. 6.

A positive curvature was observed in all isotherms, implying the formation of higher order soluble complexes at high Cyd concentrations.

Table 2

<sup>1</sup>H-chemical shifts of DM- $\beta$ -Cyd alone and in the presence of WIN 51711 in 1:2 WIN 51711/DM- $\beta$ -Cyd molar ratio



| Protons   | DM- $\beta$ -Cyd | WIN 51711–DM- $\beta$ -Cyd inclusion complex | $\Delta\delta^a$ |
|-----------|------------------|--|------------------|
| H-1       | 5.098            | 5.144  | 0.046            |
| H-2       | —                | —  | —                |
| H-3       | 3.942            | 3.886  | –0.056           |
| H-4       | 3.704            | 3.715  | 0.011            |
| H-5       | 3.860            | 3.785  | –0.075           |
| H-6       | 3.757            | 3.767  | 0.010            |
| Methyl-2' | 3.646            | 3.641  | –0.005           |
| Methyl-6' | 3.472            | 3.482  | 0.010            |

<sup>a</sup>  $\Delta\delta = \delta_{\text{complex}} - \delta_{\text{free}}$ .

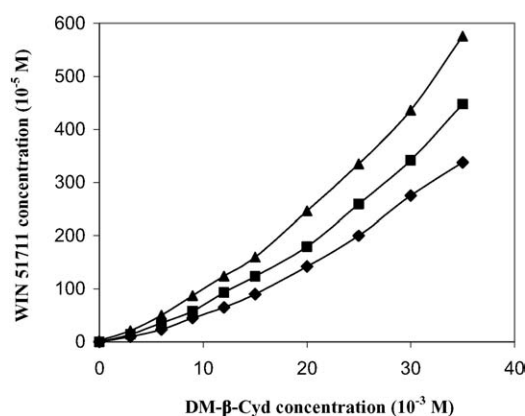


Fig. 6. Solubility phase diagrams of WIN 51711–Cyd systems at different temperatures. (♦) 5 ± 0.5 °C; (■) 15 ± 0.5 °C; (▲) 25 ± 0.5 °C.

Assuming that Ap-type isotherm results from the formation of two complexes with 1:1 and 1:2 WIN 51711/DM- $\beta$ -Cyd molar ratios, stability constants ( $K$ ) for the two complexes were determined after making a plot using the following equation [9]:

$$([S_t] - [S_0])/[L_t] = K_{1:1}[S_0] + K_{1:1}K_{1:2}[S_0][L_t]$$

where  $[S_t]$  is the total drug concentration at total Cyd concentration ( $[L_t]$ ),  $[S_0]$  is the solubility of WIN 51711 in the absence of Cyd. A plot of  $([S_t] - [S_0])/[L_t]$  vs.  $[L_t]$  results in a linear plot with an intercept of  $K_{1:1}[S_0]$  and a slope of  $K_{1:1}K_{1:2}[S_0]$ . The  $K$  values obtained at different temperatures are shown in Table 3.

The 1:1 complex was formed easier than the one at 1:2 molar ratio. High  $K_{1:1}$  values were in fact obtained at all the considered temperatures. The wide apolar internal surface of the macrocycle and the relatively low hindrance of the drug could be responsible for the high affinity between the two components and the consequent deep penetration of WIN 51711 into Cyd cavity. At increasing temperatures a reduction of both  $K_{1:1}$  and  $K_{1:2}$  values was observed, showing that complexation is an exothermal process.

Thermodynamic parameters of the binding process between WIN 51711 and DM- $\beta$ -Cyd were determined on the basis of the dependence of the  $K_{1:1}$  and  $K_{1:2}$  values on temperature. The van't Hoff plots were obtained by plotting  $\log K$  against the reciprocal of the absolute temperature. The changes in enthalpy ( $\Delta H$ ) and in entropy ( $\Delta S$ ) associated with the complexation were determined from the slope and intercept of the straight lines obtained (Table 4).

The Gibbs energy values were in all cases negative, evidencing that the formation of the inclusion complexes is energeti-

Table 3

Stability constants values ( $K_{1:1}$  and  $K_{1:2}$ ) determined for 1:1 and 1:2 WIN 51711–DM- $\beta$ -Cyd complexes in phosphate buffered solution (pH 7.0), at different temperatures

| Temperature (°C) | $K_{1:1}$ (per M) | $K_{1:2}$ (per M) |
|------------------|-------------------|-------------------|
| 5                | 31735             | 71                |
| 15               | 29010             | 55                |
| 25               | 26784             | 41                |



Table 4  
Thermodynamic parameters of WIN 51711–DM- $\beta$ -Cyd binding process

|                 | $\Delta H$<br>(cal/mol) | $\Delta S$<br>(cal/K per-mol) | $\Delta G$<br>(cal/mol) |
|-----------------|-------------------------|-------------------------------|-------------------------|
| 1:1 molar ratio | –1.396                  | 15.572                        | –5.726                  |
| 1:2 molar ratio | –4.536                  | –7.826                        | –2.354                  |

cally favored. The formation of a 1:1 inclusion complex is favored by an entropic contribution. Negligible negative  $\Delta H$  and positive  $\Delta S$  were, in fact, observed. In this way, classical hydrophobic interactions can be hypothesized as driving forces for complexation [15].

The association of the second Cyd molecule to the 1:1 formed complex was characterized by a negative  $\Delta H$  and  $\Delta S$ , probably as a result of a van der Waals interaction between host and guest. Presumably the 1:1 inclusion complex is due to the hydrophobic interaction of DM- $\beta$ -Cyd cavity and the oxazoline and phenyl moieties of WIN 51711. As a result of this interaction, less ordered water layers were formed around the complex with respect to free reactants [16].

The second DM- $\beta$ -Cyd molecule probably includes the isoxazole moiety of WIN 51711 already involved in the 1:1 complex, by means of van der Waals forces.

The negative  $\Delta H$  can be also explained by a release of enthalpy-rich water molecules from the Cyd cavity. These molecules cannot form their hydrogen-bonding potentials, thus their displacement from the cavity by suitable guest molecules with less polarity than water could result in a favorable drop in enthalpy. Negative  $\Delta S$  value could be ascribed to a more conformational rigidity of the drug due to complexation with the second Cyd molecule.

### 2.1. Solubility and kinetic stability studies of WIN 51711 with DM- $\beta$ -Cyd

The increase of water solubility and stability of the drug produced by complexation with the Cyd was evaluated in PBS solution at pH 7.0. The studies were performed using an excess of DM- $\beta$ -Cyd (1.5%, w/v) with respect to the drug.

A significant increase in drug solubility was observed, from 0.000123 mg/ml for free WIN 51711, to 0.5 mg/ml in the presence of Cyd. This trend is due to the inclusion of the drug into the cavity of a carrier with external hydrophilic characteristics, able to mask the hydrophobic properties of the drug, thus avoiding precipitation. Moreover, as a consequence of complexation, the destruction of crystalline reticule could occur.

In addition, WIN 51711 complexation with DM- $\beta$ -Cyd was effective in enhancing the stability of the drug. The effect of the Cyd was studied as a function of temperature. Stability studies were performed using unsaturated solutions of WIN 51711 containing 1.5% (w/v) of DM- $\beta$ -Cyd. Samples prepared with the same procedure used to evaluate the solubility of the drug were filtered after 2 h of stirring at room temperature and the solutions maintained at a constant temperatures (4, 10, 25, 30, and  $37 \pm 0.5$  °C) for 9 days. Degradation studies of free WIN 51711 were performed in water/methanol solution, to in-

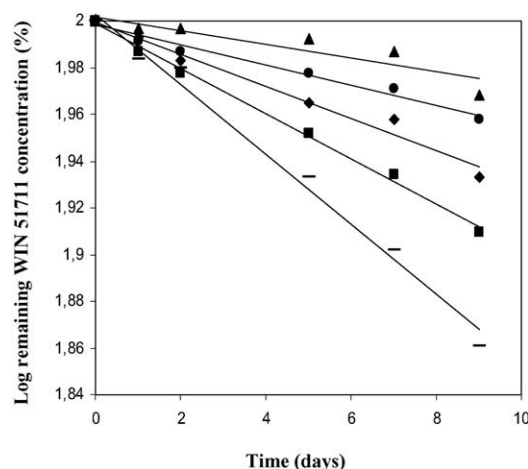


Fig. 7. First order plots for degradation of WIN 51711 in the presence of 1.5% (w/v) DM- $\beta$ -Cyd at different temperatures. (▲)  $4 \pm 0.5$  °C; (●)  $10 \pm 0.5$  °C; (◆)  $25 \pm 0.5$  °C; (■)  $30 \pm 0.5$  °C; (–)  $37 \pm 0.5$  °C.

Table 5  
Degradation rate constants ( $K_{\text{obs}}$ ) and time for 10% degradation of WIN 51711 with DM- $\beta$ -Cyd at different temperatures

| $T$ (°C) | $K_{\text{obs}}$ $10^{-3}$ (per day) | $t_{90}$ (day) |
|----------|--------------------------------------|----------------|
| 37       | 34.76                                | 3.02           |
| 30       | 22.10                                | 4.75           |
| 25       | 15.86                                | 6.61           |
| 10       | 9.58                                 | 10.95          |
| 4        | 6.87                                 | 15.27          |

crease the solubility of the drug. A complete degradation of WIN 51711 was already observed at 4 °C after 3 days.

Fig. 7 shows the first order plots obtained for degradation of WIN 51711 in the presence of DM- $\beta$ -Cyd. Kinetic stress test evidenced a linear relationship between the log of degradation constants ( $K_{\text{obs}}$ ) and the reciprocal absolute temperature. The  $K_{\text{obs}}$  and the time at which 10% WIN 51711 was degraded ( $t_{90}$ ) (Table 5) were calculated by first order kinetic equations.

An increased stability was observed for the drug in the presence of Cyd. Free drug was rapidly degraded and even at 4 °C WIN 51711 was transformed into the aminoester derivative within 36 hours. At the same temperature, in the presence of DM- $\beta$ -Cyd 90% of intact drug was still present after 15 days. It could be hypothesized that the reduced degradation of the drug in the presence of Cyd was due to the complexation of the oxazoline moiety. In this way, DM- $\beta$ -Cyd is able to exert a protective effect against the hydrolytic attack of water.

At higher temperatures the stability of the complexed drug decreased; at 37 °C WIN 51711 was 5.05 times less stable than at 4 °C. This trend can be related to  $K$  values obtained for the complexes at different temperatures. At increasing temperatures we measured lower  $K$  values and consequently higher amount of free drug in solution that can be hydrolyzed.

### 2.2. Permeation experiments through excised bovine nasal mucosa

To evaluate the effect of complexation on nasal application of the included WIN 51711, an in vitro study was performed

using excised bovine nasal mucosa as a model [17]. The mucosa sample was mounted on Franz-type diffusion cells and the receptor phase was sampled within 6 h to assay permeated WIN 51711. The membrane integrity was checked from the beginning and at the end of the experiment by transepithelial electrical resistance (TEER) measurement.

Permeation studies were performed using a solution of WIN 51711 in the presence of 1.5% (w/v) DM- $\beta$ -Cyd, compared to a suspension of the drug alone. As observed in Fig. 8, there was an increase of total WIN 51711 permeated through the mucosa when the drug was complexed with DM- $\beta$ -Cyd, from 1.24% (w/w) for the free drug to 22.14% (w/w) for the complexed drug. The free drug showed a lag time of 4 h, which was reduced to 2 h in the presence of the macrocycle.

This trend can be due to two different factors: i) the slow dissolution of solid free drug with respect to the completely dissolved WIN 51711 in the complex; ii) penetration enhancer activity exerted by the macrocycle on the biomembrane. Our previous studies using liposomes as biomembrane models demonstrated that Cyds are able to increase drug permeability through biomembranes by the extraction and complexation of the lipidic components of cells (phospholipids and cholesterol) [18].

According with the aim of this work no permeation was desirable because the drug must exert its action at a topical level only. Only a little percentage of the complexed drug was absorbed and with a latency of 2 h. In this way, the drug present in the formulation was, as requested, totally available for topical activity immediately after the application.

### 2.3. *In vivo intranasal tolerance evaluation in rabbits*

Tolerability studies were performed on rabbits after nasal administration of WIN 51711 in the presence of 1.5% (p/v) DM- $\beta$ -Cyd. An external examination of the treatment site was performed on all animals. Moreover, a gross examination was performed, including the opening of the thoracic and visceral cavities and examination of the major organs. No clinical signs attributable to treatment were observed in any animal. No ef-

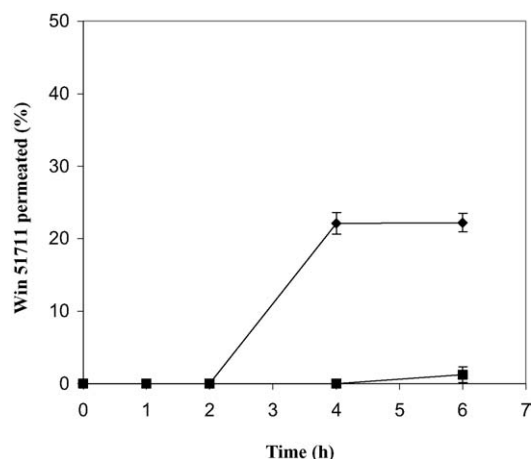


Fig. 8. Permeation profiles of WIN 51711 alone and in the presence of DM- $\beta$ -Cyd. (■) WIN 51711 alone; (◆) WIN 51711 with 1.5% (w/v) DM- $\beta$ -Cyd.

fects on body weight gain were noted. Necropsy of animals sacrificed at the end of the study showed no abnormalities. No significant change was observed at macroscopic examination that could be related to treatment. Concerning the microscopic observations, the histopathological examination of the nasal cavity, paranasal sinuses, cribriform and nasopharynx did not reveal any treatment-related changes. These results indicate that the test item is well tolerated, having no observable effect on the nasal mucosa following repeated administration.

## 3. Conclusions

The ability of DM- $\beta$ -Cyd to include WIN 51711 was evaluated both in the solid state and in aqueous solution. DSC studies evidenced an interaction between the drug and Cyd. UV spectroscopy, CD and NMR studies demonstrated a deep inclusion of the oxazoline moiety of the drug into the Cyd cavity with a partial inclusion of the phenyl ring. Isoxazole nucleus is complexed with a second Cyd molecule.

Phase-solubility studies showed Ap-type diagrams at different temperatures, implying the presence in solution of two WIN 51711–DM- $\beta$ -Cyd complexes in 1:1 and 1:2 molar ratios. The 1:1 complex was formed more easily than the other one. High  $K_{1:1}$  values were in fact obtained at all the considered temperatures.

The binding process of 1:1 WIN 51711–DM- $\beta$ -Cyd inclusion complex was favored by an entropic contribution. Classical hydrophobic interactions can be hypothesized as driving forces for complexation. The second DM- $\beta$ -Cyd molecule probably includes the isoxazole moiety of WIN 51711 already involved in the 1:1 complex, by means of van der Waals forces.

The complexation produced a significant increase of water solubility and stability of WIN 51711. In the presence of DM- $\beta$ -Cyd, 90% of intact drug was present after 15 days at 4 °C. The free drug was rapidly degraded at this temperature and within 36 h it was completely converted into the aminoester derivative.

DM- $\beta$ -Cyd induced a low permeation of WIN 51711 across excised bovine nasal mucosa with a latency of 2 h, thus allowing a topical action of the drug in the hours immediately following the application.

Concerning intranasal tolerance studies, histopathological examination of the nasal cavity, paranasal sinuses, cribriform and nasopharynx did not reveal any treatment-related changes. These results indicate that the test item is well tolerated, having no observable effect on the nasal mucosa following repeated administration.

## 4. Experimental

### 4.1. Materials

WIN 51711 was kindly provided by Angelini Pharmaceutics (Italy) and used without further purification. Heptakis-2,6-di-*O*-methyl- $\beta$ -cyclodextrin (DM- $\beta$ -Cyd) was supplied by Cy-

colab R & D Laboratory (Budapest, Hungary) and used without further purification. Polyethylene oxide (PEO 900) was supplied by Sigma. All other chemicals and solvents were of analytical reagent grade. De-ionized double-distilled water was used throughout the study.

#### 4.2. Preparation of WIN 51711–DM- $\beta$ -Cyd solid samples

The solid complexes of WIN 51711 and DM- $\beta$ -Cyd were prepared by a kneading method in 1:1 and 1:2 molar ratios. An amount of WIN 51711 (23.6 mg;  $7.5 \times 10^{-5}$  mol) and DM- $\beta$ -Cyd (100 mg;  $7.5 \times 10^{-5}$  mol or 200 mg;  $15 \times 10^{-5}$  mol) were wetted with a 50:50 (v/v) mixture of methanol/phosphate buffered solution (PBS, pH 7) and kneaded. During kneading drops of the solvent solution were added to the pastes to keep them moisturized. This procedure was carried out for 30 min. The obtained pastes were dried under reduced pressure at room temperature (22 °C) for 1 day.

#### 4.3. Differential scanning calorimetry (DSC)

DSC analysis were recorded on a Mettler DSC 12E equipped with a Haake D8-G thermocryostat. Mettler TA89E and FP89 system software was used for the data acquisition. Indium was used to calibrate the instrument. Each sample (inclusion complexes, physical mixtures in the same molar ratio, and pure components) was scanned at a speed of 5 °C/min in the 30–300 °C temperature range.

#### 4.4. UV and circular dichroism spectroscopy (CD)

UV spectra of WIN 51711 alone or in the presence of different DM- $\beta$ -Cyd concentrations (drug/Cyd mole ratio: 1:1, 1:2, 1:10, 1:40) were obtained in a methanol/PBS (pH 7) solution (50:50, v/v) with an UV/VIS Shimadzu 1601 spectrophotometer. CD spectra on the same samples were performed on a Jasco J-600D recording spectropolarimeter.

#### 4.5. NMR spectroscopy

$^1\text{H}$ -NMR spectra of free WIN 51711 or in the presence of DM- $\beta$ -Cyd in 1:2 molar ratio were carried out on a Varian Gemini 300 spectrometer at 300 MHz, at a probe temperature of 303°K. All samples were solubilized in a  $\text{D}_2\text{O}/\text{CD}_3\text{OD}$  solution (50/50, v/v). No internal standards were added to the samples due to their interaction with the Cyd cavity, the residual sign of  $\text{CD}_3\text{OD}$  at 3.3 ppm was used as reference.

#### 4.6. Solubility phase studies

Solubility phase diagrams of WIN 51711–DM- $\beta$ -Cyd system were carried out according to the method described by Higuchi and Connor (1965) [9]. An excess amount of WIN 51711 was suspended in PBS (pH 7.0) containing increasing amounts of DM- $\beta$ -Cyd ( $0\text{--}35 \times 10^{-3}$  M) and stirred for 4 h at different temperatures (5, 15 and 25 °C). The amount of WIN

51711 in solution was determined after filtration through a 0.2  $\mu\text{m}$  Millipore filter by HPLC.

Thermodynamic parameters were evaluated using the van't Hoff plot, on the basis of the dependence of the stability constant ( $K$ ) on temperature.

#### 4.7. HPLC analysis

The HPLC apparatus consisted of a Hewlett-Packard model 1050 liquid chromatograph (Hewlett-Packard, Milan, Italy), equipped with a 20  $\mu\text{l}$  Rheodyne model 7125 injection valve (Rheodyne, Cotati, CA, USA) and an UV-VIS detector. The chromatographic analysis were performed on a Lichrosphere<sup>®</sup> 100  $\text{C}_{18}$  RP column (particle size, 5  $\mu\text{m}$ ;  $125 \times 4$  mm I.D.; Merck, Darmstadt, Germany), equipped with a 5  $\mu\text{m}$  Lichrosphere<sup>®</sup> 100  $\text{C}_{18}$  RP guard column ( $4 \times 4$  mm I.D.) (Merck) and eluted isocratically at room temperature. The mobile phase consisted of a 55:45 (v/v) mixture of acetonitrile and an aqueous solution containing 40 ml/l of acetic acid and 2.6 g/l of sodium octanesulfonate (pH 2.6). Drug detection was carried out at  $\lambda_{\text{max}}$  270 nm and the flow rate was set at 1 ml/min. Calibration curve for quantitative evaluation of the drug was linear in the range 0.4–20.7  $\mu\text{g/ml}$  of WIN 51711 ( $r^2$ , 0.9998).

#### 4.8. Kinetic stability measurements

The drug (10 mg) was suspended in 20 ml of PBS (pH 7.0) containing 1.5% (w/v) of DM- $\beta$ -Cyd. The suspension was stirred at 100 rpm for 2 h at room temperature (22 °C), in the dark, until complete drug dissolution. The solution was filtered and immediately analyzed by HPLC to determine the content of WIN 51711. At this time no degradation product was observed. Aliquots of 5 ml of the drug solution were stored at different temperatures (4, 10, 25, 30 and 37 °C) for 9 days. Portions of each sample were periodically withdrawn and analyzed.

The first order degradation constants ( $K_{\text{obs}}$ ) and the time for 10% WIN 51711 degradation ( $t_{90}$ ) and for the overall degradation of the drug were determined from the slopes of the linear semilogarithmic plots of the remaining drug vs. time.

#### 4.9. Permeation experiments through excised bovine nasal mucosa

In vitro permeation studies were performed using excised bovine nasal mucosa mounted on Franz-type diffusion cells (LGA, Berkeley, CA, USA). The mucosa samples were taken by healthy animals (12–18 months of age) immediately after slaughtering and poured before use into a PBS solution containing heparin. The membrane integrity was assayed by means of transepithelial electrical resistance (TEER) measured with a voltmeter (Millicell ERS). The measured TEER was ( $42 \pm 12 \Omega \text{ cm}^2$ ). Each mucosa sample was mounted in Franz-type diffusion cells with the external face side up. The area of mucosa available for diffusion was 0.75  $\text{cm}^2$ . The receptor was filled with 4.5 ml of PBS (pH 7.0)/ethanol solution (50:50, v/v) for ensuring pseudo-sink conditions by increasing the solubility of

WIN 51711 in the receptor phase. The receptor fluid was constantly stirred with a small magnetic stirring bar to ensure homogeneity. The apparatus was thermostated at  $37 \pm 0.5$  °C throughout the experiment. Aliquots of 200  $\mu$ l of a suspension in PBS (pH 7.0) containing 0.5 mg/ml of free WIN 51711 or a PBS (pH 7.0) solution in the presence of 1.5% (w/v) DM- $\beta$ -Cyd were poured in the donor compartment. Samples of the receiving solution were withdrawn at different times during the experimental period (6 h); the sample volumes were replaced with the same amounts of fresh receptor phase. At the end of the experiments the TEER was remeasured. All samples were analyzed by HPLC to determine the concentration of WIN 51711. The obtained values were corrected for the dilution used during the sampling.

#### 4.10. *In vivo intranasal tolerance evaluation in rabbits*

*In vivo* intranasal tolerance studies were performed on a group of 12 New Zealand White rabbits (six males and six females) divided into two treatment groups each of three male and three female animals, weighing approximately 2 kg ( $2.1 \pm 0.3$  kg). The animals were supplied by Enzo Francucci, Rieti (Italy). Animal room controls were set to maintain temperature and relative humidity at  $19$  °C  $\pm$   $2$  °C and  $55\% \pm 15\%$ , respectively. There were approximately 15–20 air changes per hour and the rooms were lit by artificial light for 12 hours each day. The animals were housed individually in stainless steel cages. Drinking water was supplied *ad libitum* to each cage via water bottles. A commercially available laboratory diet (2 RB 15, Mucedola S.r.l., Settimo Milanese (MI), Italy) was offered *ad libitum* throughout the study. WIN 51711 was prepared by dissolution in a vehicle consisting of a mixture of 8% PEO 900 (Poly ethylene oxide; Sigma) and 1.5% DM- $\beta$ -Cyd in pH 7 PBS to give a concentration of 0.05 mg/ml. Control animals receiving vehicle alone.

WIN 51711 or vehicle alone were administered by nasal instillation at an approximate rate of 250  $\mu$ l/min, divided between the nostrils. The dose was administered to each animal by means of a microliter graduated syringe at a dose volume of 300  $\mu$ l/kg body weight. Animals were dosed three times daily at 4 hour intervals for a minimum of 14 consecutive days. Throughout the study, all animals were checked twice daily for overt toxicity signs and general effects related to treatment. On day 14, animals were sacrificed by intravenous injection of a suitable anesthetic agent (Tanax<sup>®</sup>, Intervet).

Samples of the tissues of the nasal cavity, paranasal sinuses, cribiform plate and nasopharynx were collected, fixed in 10% buffered formol saline, de-calcified and stained with haematoxylin and eosin and then with PAS/Alcian blue.

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