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INFLUENCE OF γ-CYCLODEXTRIN ON THE NYSTATIN RELEASE FROM OINTMENTS AND ITS ANTIFUNGAL EFFECT

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تضمن هذا البحث تحضير متراكب النيستاتين مع جاما سيكلوديكسترين ودراسة خصائص المتراكب كيميائيا وطبيعيا ومقارنته بمخلوط فيزيائى للعقار ، وقد تم تحضير نوعين من المراهم ، النوع الأول هو مرهم الامتصاص ، والنوع الثانى من المراهم المحبة للماء (زيت في ماء) على أن يحتوى كل منهم كمية محسوبة من العقار بمفرده وكمية مساوية فى المخلوط الفيزيائى وأخرى مساوية من المتراكب.

وقد تم دراسة أنطلاق العقار من المراهم بنوعيها وميكانيكية نفاذية العقار عن طريق تعيين معدل نفاذية العقار ، ووجد أن معدل النفاذية أو الإنطلاق يتبع نظام الانتشار.

وقد أكدت النتائج أن معدل إنطلاق العقار (المحضر فى صورة متراكب) من نوعى المراهم أكبر من معدل إنطلاق العقار الغير متراكب وأن معدل الإنطلاق أكبر من المراهم (زيت/ماء) عنه من المراهم الأخرى إذا قورنت النتائج بإنطلاق العقار من نوعى المرهم.

وتضمن البحث أيضا إستخدام طريقة كروماتوجرافيا السوائل ذات الضغط العالى للتعيين الكمى للعقار المنطلق من المراهم.

وقد توصلت النتائج المعملية إلى أن المتراكب المحضر مع جاما سيكلوديكسترين له تأثير واضح كمضاد للفطريات من نوع كانديدا. وبدراسة النتائج يتضح أن جاما سيكلوديكسترين زاد من معدل إنطلاق العقار من المراهم ولم يقلل من فاعليته ضد الفطريات.

The solid complex of nystatin-γ-cyclodextrin (γ-CD) in a molar ratio 1:1 was prepared. Physico-chemical characteristics of the nystatin-γ-CD complex was studied by x-ray diffraction, differential scanning calorimetry and infrared spectroscopy. Absorption and hydrophilic (o/w) ointments were prepared, containing each of the intact drug, the γ-CD complex and the physical mixture of the drug with γ-CD; respectively.

The in-vitro release of nystatin from both types of ointments was examined and the concentration of nystatin released was estimated by HPLC method using Cyclobond column for the resolution of the drug. The release kinetic mechanism was found to follow both diffusion controlled and first-order kinetic mechanisms. The solid complex-prepared showed improved release from o/w rather than absorption ointment bases as well as enhanced antifungal effect against Candida Albicans.

INTRODUCTION

Nystatin is a prominent member of the polyene macrolid antifungal antibiotics[1]. It is used to treat valvovaginal candidiasis[2] and useful in the treatment of mycotic infections used topically.

Nystatin is insoluble in water, sensitive to light, heat, oxygen and extreme pH values[3]. Tissue culture, an important tool in biotechnology, needs soluble antifungal agents[4] which have to be dissolved in the culture medium. Nystatin would be very adequate for this purpose, however it is practically insoluble in water and rapidly decomposes by oxidation. Chemical modification of nystatin or

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amphotericin - β(similar to nystatin in structure) increases the solubility but diminishes their antifungal activity.

Many literature demonstrated the effect of Cyclodextrins on the stability of drugs and improvement of solubility through complex formation[4] Van doorne *et al.*,[5] studied the complex formation of β-CyD with some anti-mycotic imidazole derivatives. The antifungal activity of these complexes was reported to be the same as of the pure drug.[6]

The usefulness of molecular entrapment of drugs with γ-Cyd in pharmaceutical formulations has been realized[7-9]. Rajagopalan *et al.*,[10] reported the complexing behavior of amphotericin -ß with γ-CyD (structure resemblance to nystatin). Furthermore, besides the solubility enhancement, stabilization is the important consequence of complexation of nystatin with γ-CyD. Stability and microbial activity of nystatin and its γ-CyD complex were studied[8]. Analysis of nystatin through titrimetric, colorimetric, electrophoretic, polarographic, chromatographic (GL, TLC) and spectrophotometric method have be reported[1]. There is a gap in literature concerning nystatin HPLC method for analysis.

The aim of the present work is to study the influence of γ-CyD on the release rate of nystatin via:

1- The preparation and characterization of nystatin -γ-CyD complex.
2- Formulation of absorption and hydrophilic ointment bases.
3- Construction of HPLC method for successful quantitative determination of nystatin released.
4- Release kinetic study .
5- Evaluation of antifungal effects.

EXPERIMENTAL

Materials:

Nystatin (EPICO Co. Egypt); γ-cyclodextrin kindly donated by Roquette, Lestrem (France) were used without further purification. McIlvaine's phosphate-citrate buffer pH 7,[11]; Polyethylene glycol 1020 (Carl Roth. Karisruhe); Glyceryl monostearate (BDH poole, England); Cetyl alcohol (Adwic. Labo. chemicals. Prolabo); Sodium lauryl sulfate (Cambrain chemicals); Mycostatin cream (From market, Squibb Co., Egypt.).

Agar (SDA) and Sabouraud Dextrose broth (SDB) was used for cultivation of the yeast and for the inhibition zone measurements. Candida albicans DS 12559 was used for inhibition zone measurements.

Equipment:

- IR (Infrared Spectrophotometer 470) Shimadzu
- x-ray diffractometer (Philips diffractometer pw 1710), (Netherlands, CuK α-radiation (1.5418), nickel filter, 50 KV, 40 mA and 0.06°/sec.
- DSC [Differential scannining calorimeter 50] connected with thermal analyzer TA-501 (shimadzu)
- High performance liquid chromatography (HPLC) consist of Gilson pump 805 and Manometric Module (France), equipped with stainless steel column (100x4.6 mm) packed with β-cyclodextrin chemically bonded to high-purity silica gel (Cyclobond I, Advanced Separation Technologies, U.S.A.). The detection was effected Spectrophotometrically at 320 nm (aufs 1) using Gilson 115 UV Detector. Chromojet integrator (Spectra physics) was used to monitor the chromatographic characteristics.
- Mechanical stirrer VEB MLW PRUFGERATE WERK MEDINGEN/SITZ FREITAL Made in GDR Type ER I0.

Methods:

- Preparation of nystatin solid complex:-

Equimolar quantities of nystatin and γ-CD were mixed with 100 ml phosphate-citrate buffer pH 7 and the suspension was stirred vigorously for 4 days at 20°C in the dark, then filtered through 0.25 mm membrane filter. The solid precipitated complex dried under reduced pressure, protected from light and heat for two days[5].

- Preparation of the physical mixture of nystatin with γ-CD:

The physical mixture was prepared by simple dry mixing of molar quantities of nystatin and γ-CD adopting the geometric method.

Characterization of the nystatin γ-CD Complex:

- Infrared absorption spectroscopy (IR):

The IR spectra were performed using the potassium bromide disk method and at a pressure of 400 Kg cm^{-2}.

- X-ray diffractometry:

The powder x-ray diffractometer was operated under the following conditions: CuK α-radiation (1 - 5418), nickel filter, voltage 50 kV, current 40 mA and scanning speed 0.06°/sec.

- Differential scanning calorimetry (DSC):

The calorimeter was operated at a scanning speed of 10°C min^{-1} between room temperature and 200°C under dry nitrogen stream as a purge gas and the sample weight being approximately 3-4 mg using Mettler M_3 microbalance.

Analysis:

- Chromatographic conditions:

Mobile phase was consisted of 0.05 M phosphate buffer (pH 7) and methanol in ratio 70:30 v/v. The mobile phase was freshly prepared and sonicated before use. The flow rate was 0.8 ml min^{-1}. The volume of injected sample was 20 μL.

Standard Solution:

Stock solution (100 mg ml^{-1}) of nystatin was prepared in methanol and serially diluted with mobile phase to give final concentration of 5-75 μg/ml.

- Determination of nystatin content:

Twenty five mg of the complex was dissolved in 250 ml phosphate - citrate buffer pH 7. After appropriate dilution; nystatin content was determined via HPLC analysis.

Table 1: Formulae of nystatin ointments

	Absorption ointment
Ingredients	% w/v
Cetylalcohol glyceryl monostearate white beeswax white petrolatum propylene glycol	3 3 8 86 12
	(O/W) Hydrophilic ointment
Cetylalcohol white petrolatum propylene glycol Sodium lauryl sulfate purified water	25 25 12 1 37

Preparation of absorption (w/o) and hydrophilic (o/w) ointments:

The formulae of the tested ointments are listed in Table 1. The ointment base was prepared by dissolving sodium lauryl sulfate in purified water and; adding propylene glycol then heating the mixture to 70°C. The oily components heated to the same temperature were added with vigorous stirring to the aqueous phase. The mixture was cooled to room temperature with stirring by mechanical stirrer until homogeneous cool ointment was obtained. An equivalent amount of nystatin-powder to 0.375 g, its physical mixture or the complex was incorporated in polyethylene glycol 400 to ensure uniform distribution, then

added the remainder-part of the cool base and mixed thoroughly. The obtained ointments were stored in the refrigerator until the beginning of release experiments on the next day.

Permeation study:

Permeation behavior of nystatin through cellophane membrane (type 36/32 visking) using open ended glass tube of cross-sectional area 4.91 cm^2 as donor compartment. The cellophane membrane was soaked in distilled water 12 h then washed with phosphate-citrate buffer pH 7 before use. The membrane was fixed to one end of the tube. One gram of nystatin ointment

(equivalent to 100000 U) in the absence and in the presence of γ-CD as physical mixture or nystatin -γ-CD complex was placed onto the donor tube.

Five ml of the buffer was transferred into the tube which is suspended in 250-ml beaker containing 150 ml phosphate-citrate buffer pH 7 (receptor compartment). The whole assembly was placed in a shaking water bath maintained at 37°C ± 1°C and a rate of 50 rpm. Five ml of the release medium was withdrawn at specified time intervals. The withdrawn volume was compensated with fresh medium. The samples withdrawn were analyzed by HPLC for estimation of the nystatin concentration released. All release experiments were done in triplicate, the samples were protected from light allover the time of experiment. The amount of released nystatin per unit surface area was plotted against the square-root of time and the log percent remainder of nystatin was plotted against time, The release rate was calculated from the straight line obtained. The apparent dialytic rate constant (K), was calculated as follows[12]:

$$K = \frac{-(Slope)\ (2.3)\ (V_i V_o)}{V_i + V_o}$$

where V_i is the volume of the test medium in the dialysis tube and V_o is the volume of the test medium outside the dialysis tube.

Effect of Complexation with γ-CD on the antifungal activity:

The assays were carried out according to Drouhet *et al.*,[13]. Candida Albicans was cultured for 24 h. Inhibition zones were read after overnight incubation at 37°C. The results in terms of average diameter inhibitory zones of nystatin are summarized in Table 6.

RESULTS AND DISCUSSION

Characterization of inclusion Complexation:

Different instrumental techniques were used to examine and characterize complexation of nystatin and γ-CD.

Figure 1 shows the x-ray diffraction pattern of nystatin -γ-CD complex as well as drug. It is evident from the figure that the x-ray pattern of nystatin-γ-CD has different peaks than drug which indicates that the crystallinity pattern was changed. This is a direct proof of the formation of different crystalline structure and can be considered as indirect proof of the complex formation.

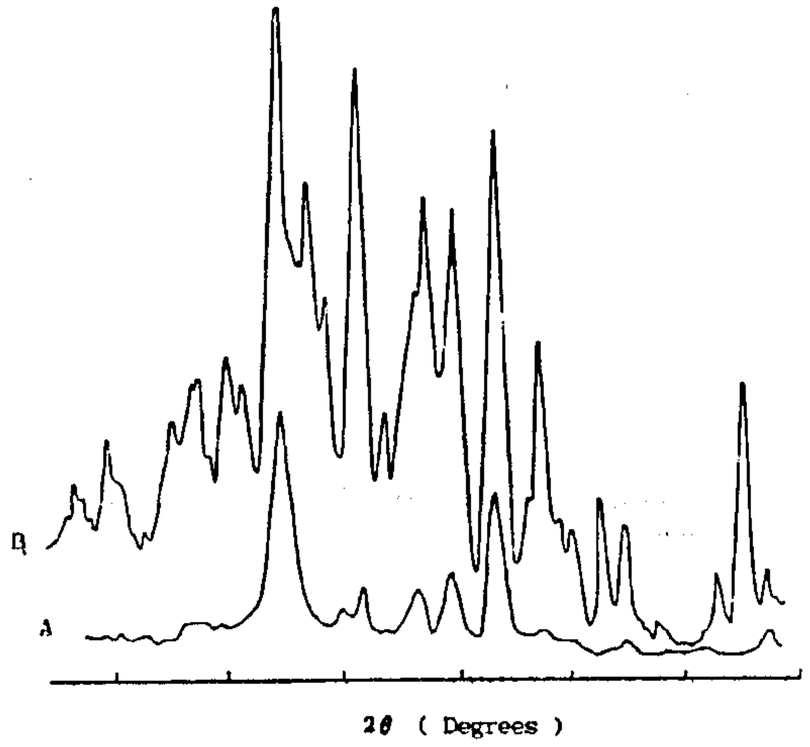

Fig. 1: Powder X-ray diffraction pattern for nystatin (A) and a 1:1 molar ratio physical mixture of nystatin-γ-CD (B).

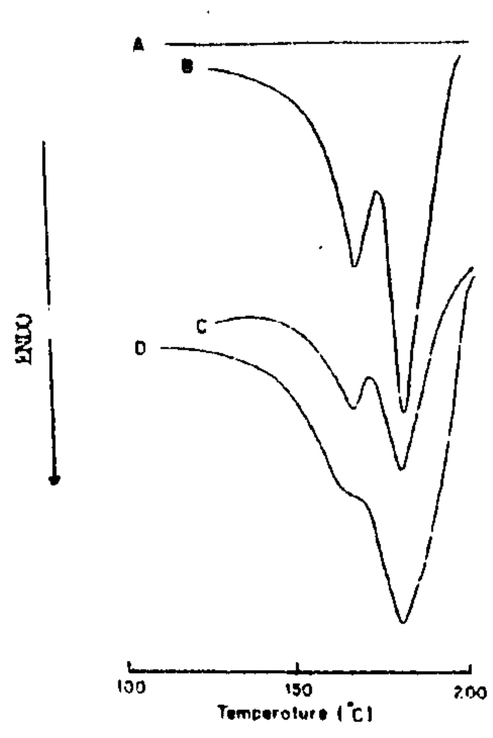

Fig. 2: The DSC thermogram of γ-CD (A); nystatin (B); a 1:1 molar ratio physical mixture of nystatin-γ-CD (C) and a 1:1 molar ratio inclusion complex of nystatin-γ-CD (D).

From the differential scanning calorimetry (DSC) thermograms (Fig. 2) nystatin exhibits its characteristic thermal endothermic peak at 165°C and 179°C which is due to the

decomposition of the drug instead of melting. The interaction of nystatin with γ-CD is confirmed by disappearance of the peak at 165°C.

The infrared absorption spectra of nystatin, γ-CD, nystatin γ-CD complex and nystatin-γ-CD physical mixture is presented in Figure 3. The spectra exhibited apparent changes specially in the region of 1700-1500 cm^{-1}.

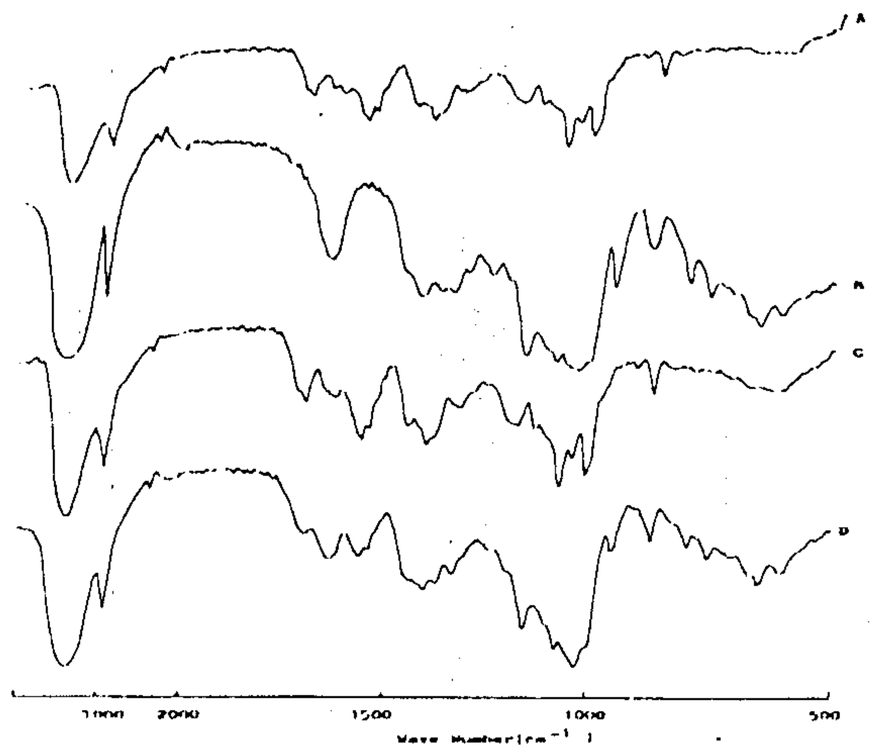

Fig. 3: IR spectra of nystatin (A); γ-CD (B); a 1:1 molar ratio inclusion complex of nystatin-γ-CD (C) and a 1:1 molar ratio physical mixture of nystatin-γ-CD (D).

HPLC Analysis:

The strength of interaction of β-CD with nystatin is weak and observed from elution order. During the development of the mobile phase, it was found that the resolution of nystatin was dependent on the content of phosphate buffer of the mobile phase which consisting of phosphate buffer pH 7: methanol (70:30 v/v).

Under the specified HPLC experimental conditions, the peak area measured for nystatin was directly related to the concentration used in the range 5-75 mg ml^{-1} (standard solution). This dependence is strictly linear as depicted from the regression analysis data given in Table 2.

Table 2: Calibration data for standard solution

	Conc. mg ml^{-1}	Intercept	slope	Corr. Coeff.r* (SD)**
Nystatin	5-75	0.258	0.396	0.997 (±0.012)

The developed HPLC method proved simple, successful, accurate and precise one.

As revealed from Figure 4, nystatin would be analyzed with good separation. There was no indication that ointment bases interfered with the separated peak.

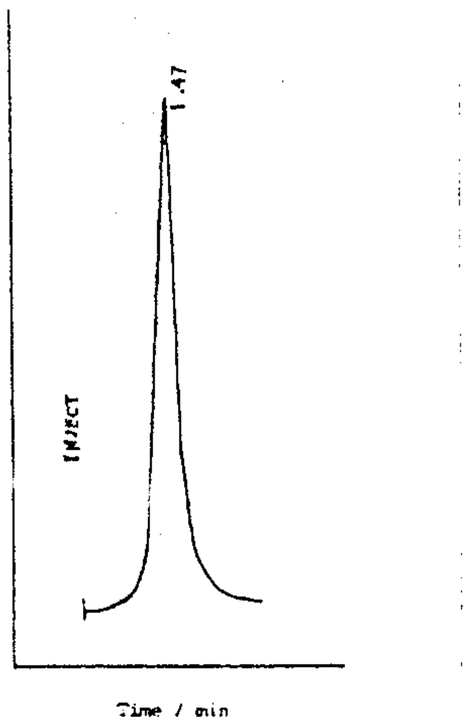

Fig. 4: Chromatogram of nystatin

Drug release from ointment bases:

The release behavior of nystatin from w/o ointment bases was compared with that of nystatin complexed with γ-CD and its physical mixture. Fig. 5 (a and b) show the release profile of nystatin depicted as the amount released per unit surface area against the square root of time. Table 3 illustrates the kinetic analysis of the release data calculated as Higuchi model and first-order Kinetic. It is evident that the release rate of nystatin was significantly increased by complexation, particularly from hydrophilic (o/w) ointment. The linearity of the plots with high correlation coefficients for both mechanisms and manipulation may indicate that the release of nystatin followed both mechanisms; first-order kinetic and diffusion-controlled[14]. The release rate of nystatin from absorption ointment was higher than that from o/w hydrophilic ointment due to the hydrophobic properties of the drug and higher solubility towards lipid phase as shown from Table 4.

The dialytic-rate constant illustrated in (Table 5) indicated that complexation with γ-CD had significant effect on dialytic rate of nystatin. The higher release rate was nystatin complexed with γ-CD from ointments was due to the

improving effect of γ-CD on the aqueous solubility of the drug and the decrease in its crystallinity. The order of increased release rate of nystatin corresponds to the following: nystatin < physical mixture < nystatin γ-CD complex.

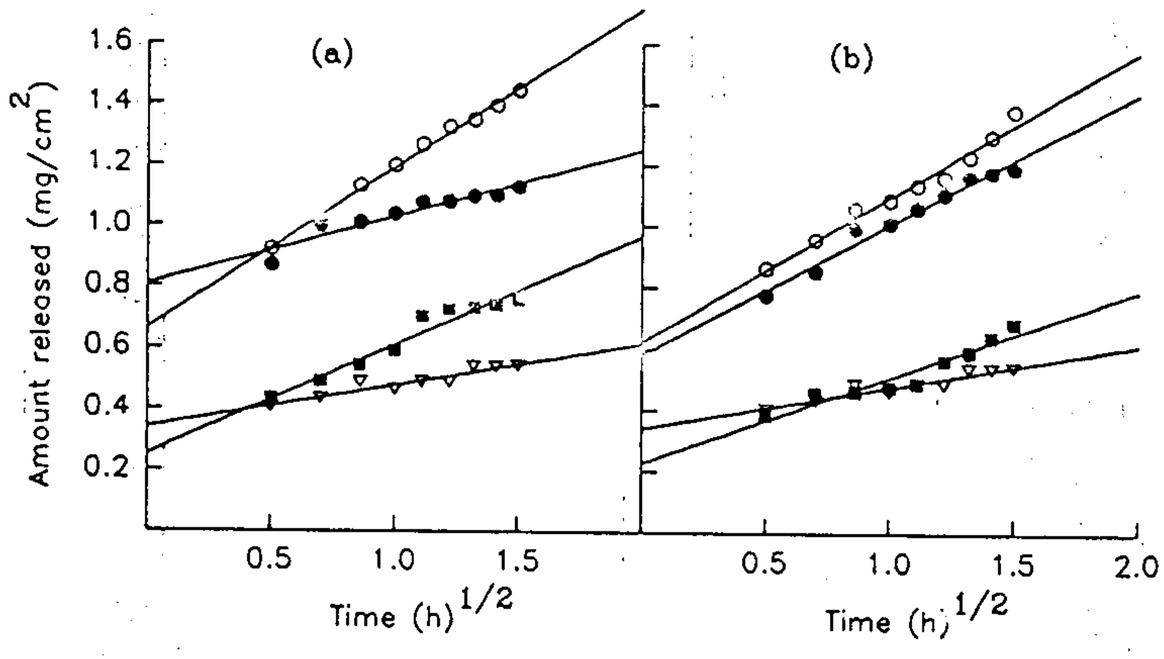

Fig. 5: Release of nystatin as a function of the square root of time from 0.024% w/w a) Hydrophilic ointment b) absorption ointment o nystatin/ CD; ▪ nystatin/ CD physical mixture; • nystatin only; ▿ marketed nystatin ointment.

Table 3: Release characteristics of nystatin complexed with γ-cyclodextrin from absorption and hydrophilic ointment.

Type of ointment	*Corr. coeff. (r)	**Corr. coeff. (r)	Slope (k)	Intercept (h)	T½ (h)	Lag time (h)
Nystatin Hydro.	0.6504	0.9332	0.2149	0.8160	3.2248	3.797
Nystatin γCD complex	0.9956	0.9885	0.5138	0.6749	1.3482	1.314
Nystatin/physical mixture	0.9743	0.9734	0.3567	0.2540	1.9428	0.712
Nystatin absorp	0.9867	0.9827	0.4298	0.5814	1.6124	1.353
Nystatin/γCD complex	0.9873	0.9948	0.4784	0.6216	1.4486	1.299
Nystatin/physical mixture	0.9569	0.9882	0.2812	0.2297	2.4644	0.817
Marketed nystatin	0.9859	0.9323	0.1381	0.3333	5.0181	0.414

* Diffusion - Controlled.

** First - order kinetic.

Table 4: Solubility of Nystatin in ointment ingredients

Ingreients of ointment	Solubility conc. (mg)
Glyceryl monostearate	35 (± 0.51)
Cetylalcohol	66 (± 0.22)
Propylene glycol	88 (± 0.15)
White beeswax	90 (± 0.12)

Mean of four readings (n=4).

Table 5: Effect of γ-cyclodextrin on the dialytic rate constant of nystatin from ointments.

Type of ointments	Apparent dialytic rate constant K (min^{-1})
Nystatin hydrophilic oint.	2.3916
Nystatin γ-CD hydro. oint.	5.7181
Nystatin/physical mixture hydro. oint.	3.9797
Nystatin absorption oint. w/o	4.7832
Nystatin γ-CD absorp oint.	5.3241
Nystatin/physical mixture absorp. oint.	3.1295

Table 6: Effect of γ-cyclodextrin on the antifungal activity of nystatin from ointments

Tested ointments	Mean inhibition zone diameter (mm.)						control
Concentration γ/ml	800	400	200	100	50	20	
Nystatin γ-CD hydrophilic ointment	15.5	14.0	12.0	10.0	5.5	2.0	0.0
Nystatin γ-CD absorption ointment	17.0	15.0	13.5	12.0	7.0	3.0	0.0
Nystatin hydrophilic ointment	15.0	14.0	13.0	12.0	7.0	4.0	0.0
Nystatin absorption ointment	16.0	15.0	14.0	13.0	8.0	4.0	0.0
Nystatin/physical mixture/hydro.oint.	10.0	9.5	7.0	6.0	3.0	2.0	0.0
Nystatin/physical mixture/absorp.oint.	11.0	8.5	7.0	6.5	4.0	2.0	0.0

* The results are average of six.

Effect of Complexation with γ-CD on the antifungal activity:

The drug γ-CD complex showed higher antifungal activity than the respective drug alone or its physical mixture (Table 6). Thus some correlation can be found between the dialytic rate constant of the drug and its microbiological activity. γ-cyclodextrin is suitable partner for nystatin, being able to improve its pharmaco-technical characteristics. A similar positive effect was reported by Van Doorne and coworkers[6-15] who obtained larger inhibition zones with anti-

mycotic imidazole derivatives when these were combined with γ-CD in topical preparations.

CONCLUSIONS

Complexation with γ-CD enhances the release properties of nystatin from hydrophilic ointments. The amorphous state of the drug plays a fundamental role in this improvement. The order of microbiological activity of nystatin against C.albicans Corresponds to the order of their release properties i.e. drug-physical mixture < free-drug < drug/ γ-CD complex.

Nystatin γ-CD complex are thus particularly worthy of attention in the context of the development of effective antifungal formulations.

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