CHAPTER 4: STABILITY OF NATAMYCIN AND ITS CYCLODEXTRIN INCLUSION COMPLEXES IN AQUEOUS SOLUTION

ABSTRACT

Aqueous solutions of natamycin and its β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin, and γ-cyclodextrin (γ-CD) inclusion complexes were completely degraded after 24 hours of exposure to 1000 lux fluorescent lighting at 4 °C. After 14 days of storage in darkness at 4 °C, 92.2% of natamycin remained in active form. Natamycin:β-CD complex and natamycin:γ-CD complex were significantly more stable ($p < 0.05$) than natamycin in its free state in aqueous solutions stored in darkness at 4 °C. Clear poly(ethylene terephthalate) packaging with a UV light absorber allowed 85.0% natamycin to remain after 14 days of storage under 1000 lux fluorescent lighting at 4 °C. Natamycin:cyclodextrin complexes can be dissociated for analysis in methanol-water-acetic acid, 60:40:5, v/v/v. Natamycin and its complexes in dissociated form were quantified by reverse phase HPLC with detection by photodiode array at 304 nm.

KEYWORDS

natamycin, pimaricin, polyene macrolide, antifungal, preservative, cyclodextrin, inclusion complex, stability, photodegradation, UV absorber

INTRODUCTION

The polyene macrolide antibiotics are very sensitive to ultraviolet light relative to other antimycotics (*1*). Natamycin is one such tetraene that is a broad spectrum antifungal employed to extend the shelf life of cheese. It is applied on cheese by dipping or spraying with an aqueous suspension or as a dry mixture with an anticaking agent. During the ripening and storage of cheese products, natamycin is decomposed but the rate of degradation remains uncertain (*2*). Cheese products are exposed to high-intensity fluorescent lighting in the retail dairy case, until consumer purchase and storage. The UV light emitted from the fluorescent lamps impacts the cheese product through the translucent sections of the polymer packaging. Shredded cheese, with its significantly increased surface area, receives a greater dosage of UV light exposure. Critical amounts of natamycin treated onto these cheese products are likely degraded by the time of purchase by the consumer.

The photolysis of natamycin in methanolic solution is very rapid upon light exposure to a xenon lamp with a spectrum similar to natural sunlight (*3*). UV irradiation exposure was clearly the most damaging treatment to decrease the biological activity of natamycin compared to exposure to air oxidation and ferrous ions (*4*).

The stability of natamycin is also affected by extreme pH conditions, heat exposure, and oxidation. At low pH values, natamycin is rapidly degraded producing mycosamine by hydrolysis of the glycosidic bond (*5*). At high pH values, the lactone is rapidly saponified, forming the biologically inactive natamycoic acid (*6*). However, natamycin is a stable compound in its trihydrate form when it is protected from both light and moisture. Several years of storage as a dry powder at room temperature results in only a few percent loss of activity (*6*). A moderate reduction in biological activity was observed after a typical heat sterilization process (*7*). Oxidative inactivation of natamycin has also been reported (*8,9*).

Cyclodextrins (CDs) act as host molecules to form inclusion complexes with a wide variety of guest molecules. The CD molecule can at least partially shield the guest molecule from degradation caused by light, oxidation, heat, and acidic or alkaline conditions. Labile guests can be insulated from a potentially corrosive environment and, therefore, reduce or even prevent drug hydrolysis, oxidation, steric rearrangement, isomerization, polymerization, and even enzymatic decomposition of drugs (*10*).

The stability of a few polyene macrolide antibiotics has been increased upon complexation with γ-cyclodextrin (γ-CD). The degradation of amphotericin B was significantly suppressed at both high and low pH extremes by complexation with γ-CD (*11*). Aqueous solutions of nystatin in the pH range 5 to 8 showed stability increases upon addition of γ-CD (*12*). The thermal stability of flavofungin dramatically increased when it was complexed with γ-CD (*13*).

A better understanding of the influence of UV light emission from fluorescent lamps on the stability of natamycin is necessary to ensure that the natamycin on the cheese surface remains in its intact, active form. The purpose of this study was to determine the stability of natamycin and its inclusion complexes with β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP β-CD), and γ-CD in aqueous solution.

MATERIALS AND METHODS

Materials. Natamycin of 90.5% purity was supplied by DSM Food Specialties (Delft, The Netherlands). USP Reference Standard Natamycin of 91.7% purity (U.S. Pharmacopeia, Rockville, MD) was used as an external standard in HPLC analysis. β-CD, food grade; HP β-CD, pharmaceutical grade (5.3-5.4 DS); and γ-CD were provided by Cerestar (Hammond, IN). High purity water was prepared with a Corning Mega-Pure System MP-6A (Corning, NY). High barrier poly(ethylene) (PE) bags with ethylene vinyl alcohol and poly(ethylene terephthalate) (PET) bags were supplied by Cryovac Sealed Air Corporation (Duncan, SC). A PET bottle (Owens-Illinois, Perrysburg, OH) and a five-layer PET bottle containing a UV absorber (Continental PET Technologies, Florence, KY) were obtained. Preparation of inclusion complexes of natamycin with β-CD, HP β-CD, and γ-CD was performed as described previously (*14*).

HPLC Analyses. Analyses were performed with a Waters 717 plus Autosampler, Waters 600 Controller, and Waters 996 Photodiode Array Detector (PDA) (Waters Corp., Milford, MA). A 4.6 x 150 mm, 5μm Waters Spherisorb C8 reverse phase analytical column, equipped with a 4.6 x 10 mm, 5μm Waters Spherisorb C8 guard column was used at ambient temperature. Three mobile phase systems were employed: (A) methanol-water-acetic acid, 60:40:5, v/v/v, (B) 100% water, and (C) 100% methanol. Sample vials were held in the autosampler protected from light

at a temperature of 4 °C. The samples were eluted in isocratic mode in mobile phase A for 20 min. The flow rate was 1.0 mL/min, the injection volume was 20 μL, and the detection wavelength by PDA was 304 nm. The wavelength range of 260-360 nm was detected by PDA. Due to the very limited solubility of β-CD in methanol-water mixtures (*15,16*) and the practical insolubility of natamycin in water, the system was designed to undergo a series of purges. A linear gradient to mobile phase B was applied from 20 to 25 min and held at 100% B until 30 min to ensure removal of any residual CD from the system. A linear gradient to mobile phase C was applied from 30 to 35 min and held at 100% B until 40 min to ensure removal of any residual natamycin. Finally, a linear gradient back to mobile phase A was applied and the system was allowed to re-equilibrate at 100% A for five minutes prior to next injection.

An external standard curve was constructed using USP Reference Standard Natamycin to quantify free natamycin content in all samples. Waters Millennium®[32] software version 3.20 was used for data management.

Treatment of Samples. Aqueous solutions of 20 mg/L natamycin and natamycin content in the respective β-CD, HP β-CD, and γ-CD complexes were prepared (n = 3). These solutions were then transferred to PE bags and packaged under two different atmospheric conditions: 1) air and 2) modified atmosphere packaging (MAP). MAP gas composition was 75% N_2 and 25% CO_2. Samples were MAP using a proportional gas blender (Smith Equipment, Model 299-037F, Watertown, SD) and vacuum packaging machine (Koch Supplies Inc., Model X200, Kansas City, MO). N_2 and CO_2 concentration in the packages was verified by a headspace oxygen/carbon dioxide analyzer (Illinois Instruments, Inc., Model 6600, Ingleside, IL). Gas samples were drawn by a hypodermic needle through a self-sealing rubber septum adhered to each package and injected into the analyzer. Sample solutions were not ultrasonicated or bubbled with gas to remove any dissolved oxygen.

These samples were stored at 4 °C with half of the bags under fluorescent lighting and the other half in darkness. The samples were illuminated by cool white 40-watt T12 fluorescent bulbs (GE Lighting, Cleveland, OH) mounted 16 cm above the samples. The samples were oriented to receive an illumination intensity of 1000 ± 50 lux. All light intensity measurements were performed with a Foot Candle/Lux Meter (Extech Instruments, Waltham, MA). At time intervals of 0, 1, 3, 7, and 14 days, the appropriate samples were removed from storage and

transferred to amber glass vials for analysis. Every effort was made to limit light exposure during transit time to analysis by HPLC.

Spectral Overlay of Polymer Packaging. A spectral scan of a natamycin sample in water between the wavelengths of 250 and 400 nm was performed on a Shimadzu UV-2101PC UV-VIS Scanning Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). Side wall cutouts of a PET bag (PET 1), a PET bottle (PET 2) and a five-layer PET bottle containing a UV absorber (PET + UV Absorber) were prepared. Each thin strip of polymer was placed in a UV cuvette and qualitatively analyzed by a spectral scan between 250 and 400 nm.

Aqueous solutions of 20 mg/L natamycin were added to these three packaging materials: PET 1, PET 2, and PET + UV Absorber. These samples (n = 1) were exposed to 1000 lux fluorescent lighting at 4 °C as described previously and quantified for remaining natamycin content by HPLC.

Statistical Analysis. Due to rapid degradation of natamycin under fluorescent lighting, no statistical analysis was necessary for light-exposed samples. Only samples stored in darkness were statistically analyzed. The sample order of HPLC analysis was randomized when loading the autosampler. Statistical analysis of the registered variables was performed by the general linear model supported by SAS (Version 8.12, 2001, SAS Institute, Inc., Cary, NC). Multiple comparisons were adjusted for the Tukey-Kramer method of the general linear model procedure to make statistical comparisons. Significant interactions were analyzed using the slicing function of the general linear model procedure. Effects were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Dissociation of Complexes. At the low pH of mobile phase A, both the amino and carboxyl groups of natamycin are protonated, yielding a net positive charge to the molecule. Neutral compounds have larger complex stability constants than the corresponding protonated or ionized species (*17*). Additionally, organic solvents usually decrease CD complex stability relative to pure water. The most commonly proposed idea for this behavior is that increasing the organic content of the aqueous mixture decreases the hydrophobic driving force, which is a major contributor to the stability of the complex in water (*18*). Therefore, natamycin as the guest

molecule appeared completely dissociated from the CD when dissolved in mobile phase A. This was further indicated by the lack of any red shift in the UV absorbance spectrum of natamycin when its complexes with β-CD, HP β-CD, and γ-CD were dissolved in the mobile phase.

Aqueous Stability in Darkness. The stability of natamycin and its CD complexes statistically differed when averaged across the storage time. Concentration decreased as storage time increased, thus, natamycin and its CD complexes degrade over time at 4 °C when stored in darkness. There was no significant difference when storing natamycin and its CD complexes in air or a modified atmosphere of 75% N_2 and 25% CO_2. The interaction of natamycin formulation and storage time was significant with different formulation types degrading at different rates over time.

β-CD:natamycin complex and γ-CD:natamycin complex were significantly more stable than natamycin in its free state (Figure 1). HP β-CD:natamycin complex does not significantly increase the stability of free natamycin. At 7 days of storage, a significant difference among formulations first appears. Between 7 and 14 days of storage, there is an increase in the degradation rate of all formulations to varying degrees. Natamycin and its HP β-CD complex appear to have faster degradation rates compared to natamycin complexes of β-CD and γ-CD. Natamycin is considered to be relatively stable in aqueous solution when stored in the dark at 4 °C since 92.2% of natamycin remained after 14 days.

Methanolic Stability in Darkness. The international standard for determining the natamycin content of cheese uses mobile phase A (methanol-water-acetic acid, 60:40:5, v/v/v). Methanol is also the solvent used to extract natamycin from cheese and cheese rind, however, this standard mentions that natamycin is unstable in aqueous methanol (*19-21*). More recent reports have stated that solutions of natamycin in aqueous or pure methanol are stable (*22,23*). In agreement with more recent studies, a 200 mg/L stock solution of natamycin in pure methanol was not found to have any apparent degradation after 24 hours of storage in darkness at room temperature. It is likely that these previous reports of natamycin instability in methanol were simply the result of inadequate protection from light.

Aqueous Stability under Fluorescent Lighting. The 1000 lux intensity of the fluorescent lighting employed in this study is typical of the exposure of products in dairy cases in grocery stores (*24*). PE packaging does not have any substantial absorption of UV irradiation, so these sample bags were exposed to the complete spectral power distribution of the fluorescent lamps. After 24 hours of 1000 lux fluorescent light exposure, 20 mg/L aqueous solutions of natamycin in its free and complexed states with β-CD, HP β-CD, and γ-CD were completely degraded.

Spectral Overlay of Polymer Packaging. Photodegradation of natamycin is greatly influenced by the wavelength of absorbed light (*25*). Visible light does not degrade natamycin (*8,26*) but rapid inactivation of natamycin is caused by UV irradiation primarily with wavelengths between 300 to 350 nm (*8*). The addition of sodium potassium chlorophyllin, which has considerable absorption in the UV wavelength range from 300 to 400 nm, has been shown to prevent inactivation of natamycin by UV irradiation (*8*).

The photostabilization principle of spectral overlay involves protecting a photolabile compound by addition of a substance that absorbs light in a similar wavelength range (*1*). While typically added as a protective excipient directly to a formulation, packaging materials can also be used to block destructive UV irradiation. Three types of clear PET packaging were examined for their ability to filter specific wavelengths of UV irradiation. In Figure 2, the UV absorption spectra in the range of 250 to 400 nm of these PET packaging materials are displayed relative to that of natamycin in water. These polymers have different UV cut-off wavelengths (< 1% transmittance) of 308 nm for PET 1, 318 nm for PET 2, and 363 nm for PET + UV Absorber.

Figure 3 shows that the degradation of aqueous solutions of 20 mg/L natamycin in PET 1 and PET 2 packaging remains very rapid under 1000 lux fluorescent lighting. Natamycin stored in PET 1 and PET 2 packaging was almost completely inactivated after 1 day and 7 days of exposure, respectively. However, an exceptional increase in the photostability of natamycin was discovered in the PET + UV Absorber packaging. After 14 days of continuous exposure to 1000 lux fluorescent lighting, 85.0% of the natamycin in this package remains intact in aqueous solution.

Photodegradation Products. The photodegradation products of natamycin are all more polar than natamycin since they elute earlier under the chromatographic conditions. In Figure 4,

pure natamycin elutes at 13.0 min and its photodegradation products have a prominent peak at 10.7 min. This broad irregularly shaped peak is likely a mixture of several different degradation products. The PDA detection in the range 260-360 nm allowed the degradation peak at 10.7 min to be characterized as a typical tetraene UV spectrum, which was identical to that of intact natamycin. The photodegradation process yields several products that contain the tetraene chromophore but are not of the original structure of the parent natamycin. The photolytic mechanism appears to have greater complexity than the simple *cis-trans* isomerization proposed by Dekker and Ark (*8*), which leads to biological inactivation.

The greater selectivity of the HPLC method appears a necessity for quantifying the amount of natamycin that remains on cheese, which has been exposed to fluorescent lighting. In agreement with Dekker and Ark (*8*), the UV absorption spectrum of natamycin may only be partially reduced after inactivation by UV irradiation. Therefore, the UV spectrophotometric method (*19-21*) will falsely indicate the presence of active natamycin and should be discontinued as a method to monitor the stability of natamycin that is exposed to a UV irradiation source.

Improved Antifungal Effectiveness. This study calls into question the current effectiveness of natamycin treated onto cheese products that are stored under high-intensity fluorescent lighting. Although, by incorporating an appropriate UV absorber into the polymer packaging, natamycin can be greatly protected while preserving the clarity of the package for consumers to view the product. The formation of inclusion complexes of natamycin with either β-CD or γ-CD allows increased stability compared to free natamycin. By combining these two technologies, very dramatic increases in the stability of natamycin can ensure that the antifungal remains in its intact, active form on the cheese surface throughout the product shelf life.

ABBREVIATIONS USED

CD, cyclodextrin; β-CD, β-cyclodextrin; HP β-CD, hydroxypropyl β-cyclodextrin; γ-CD, γ-cyclodextrin; DS, degree of substitution; PE, poly(ethylene); PET, poly(ethylene terephthalate).

ACKNOWLEDGMENT

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FIGURES

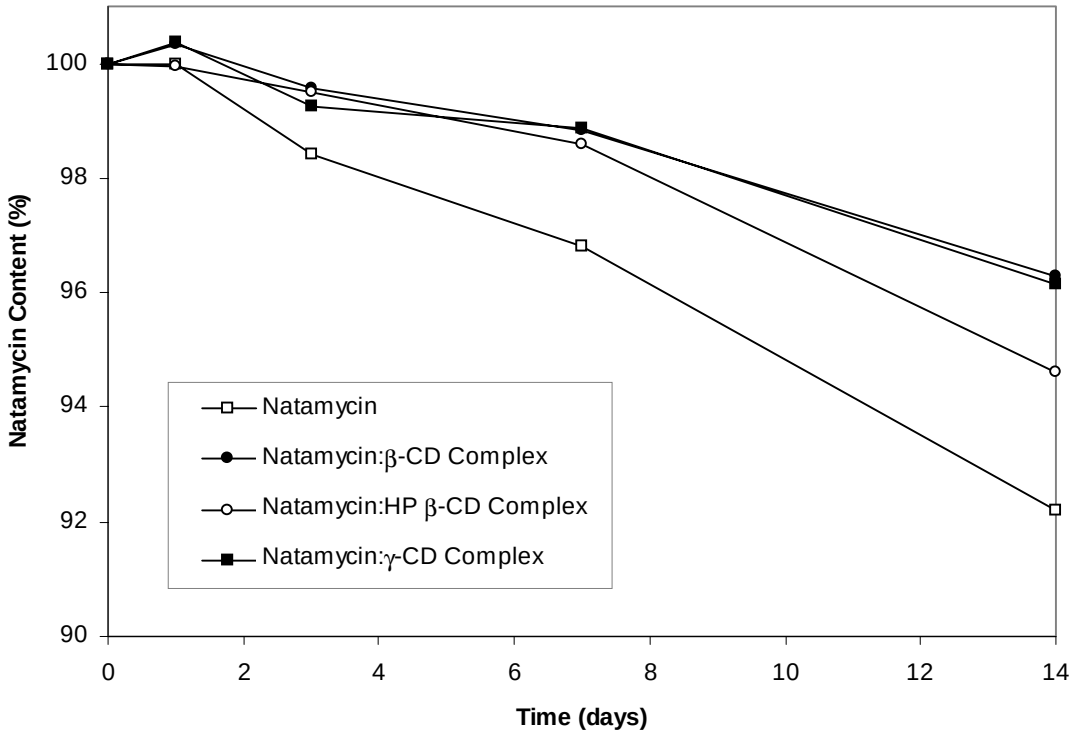

Figure 1. Degradation of 20 mg/L aqueous solutions of natamycin in free and cyclodextrin (CD) complexed states while stored in the dark at 4 °C.

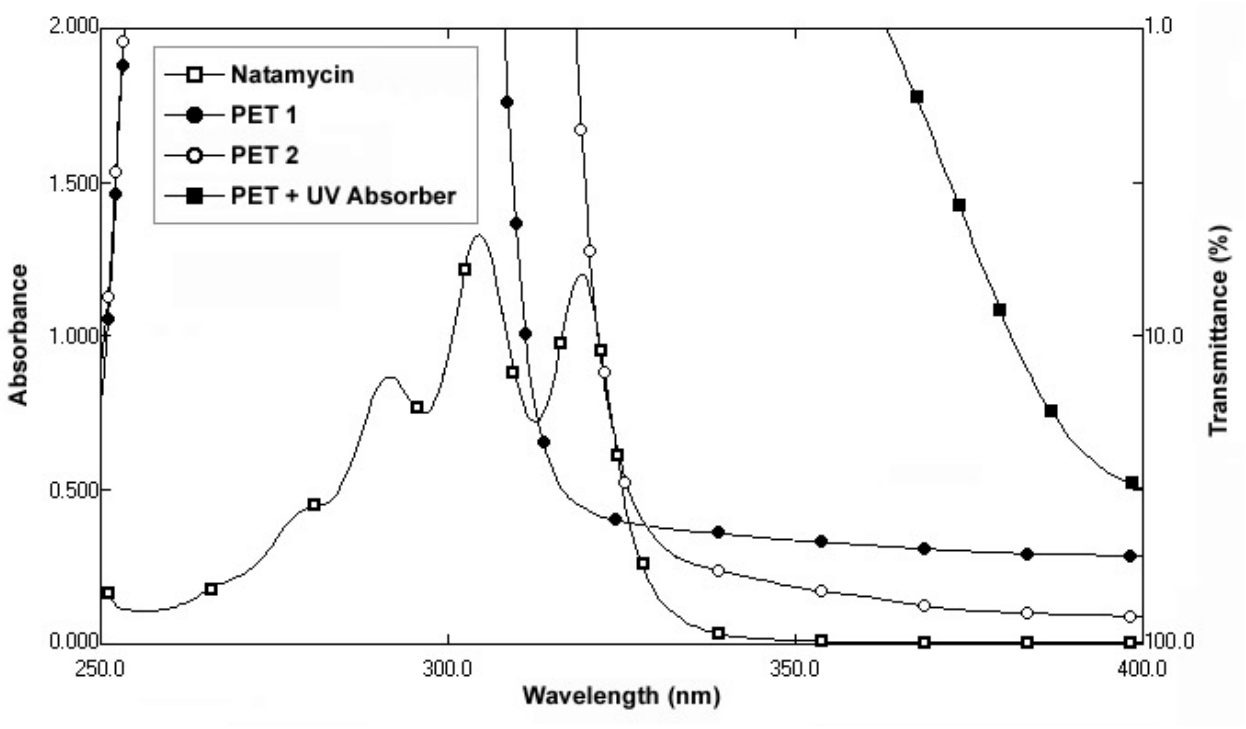

Figure 2. UV absorption spectra of natamycin in water compared with spectra of different types of PET packaging films.

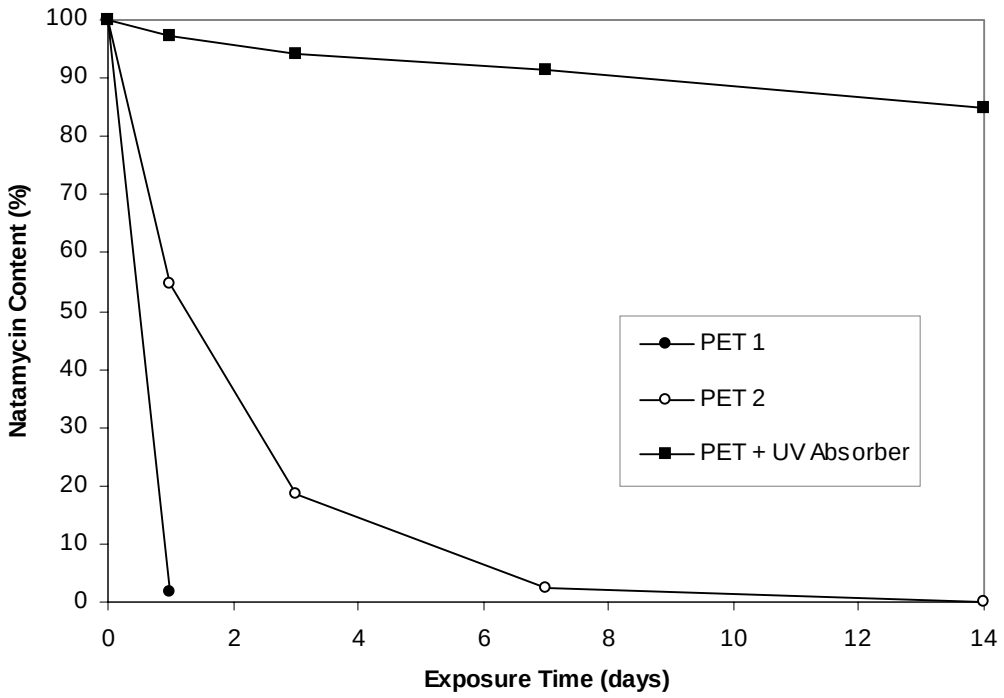

Figure 3. Stability of 20 mg/L aqueous solutions of natamycin exposed to fluorescent lighting of 1000 lux while stored in different types of clear PET packaging at 4 °C.

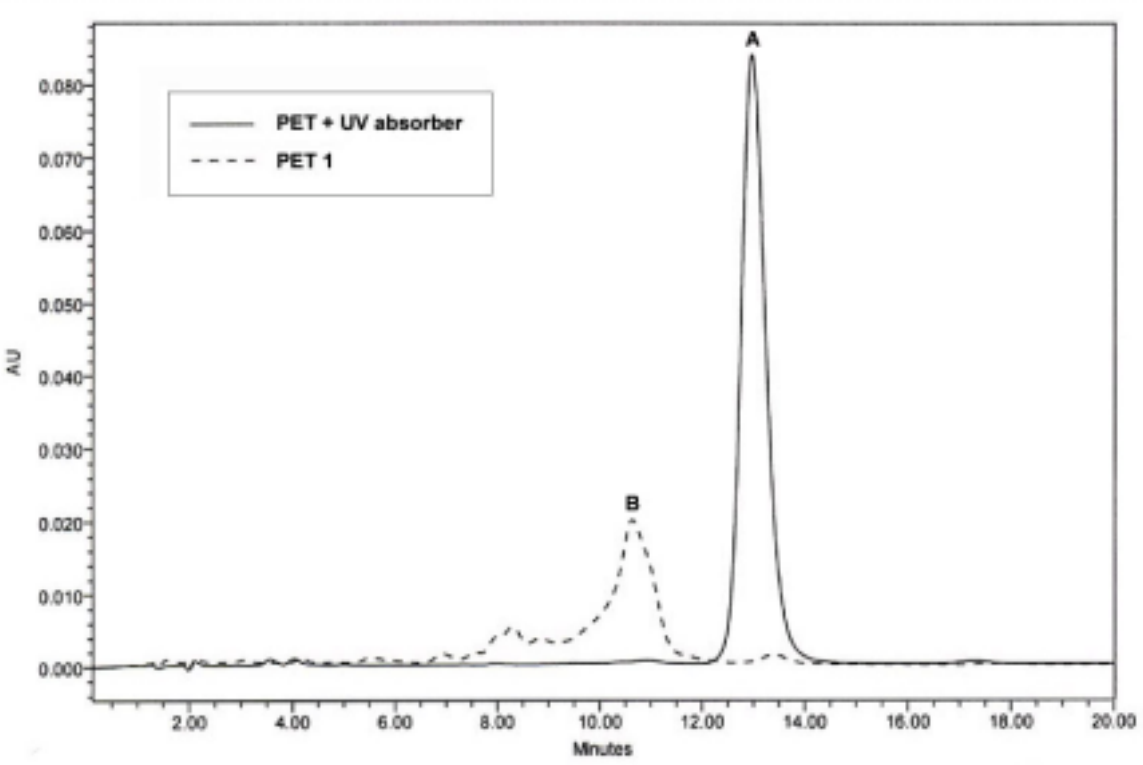

Figure 4. HPLC chromatograms of aqueous solutions of natamycin after 24-hour exposure to fluorescent lighting of 1000 lux and storage at 4 °C in different types of clear PET packaging. Detection: UV absorbance at 304 nm. Peaks: A = natamycin, B = photodegradation products.