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LC DETERMINATION OF NATAMYCIN IN DOOGH WITH UV DETECTION

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Abstract

A rapid and simple High-Performance Liquid Chromatographic method with UV detection (HPLC-UV) has been developed for the first time to determination of natamycin in Doogh. Doogh is a refreshing drink that is made with yogurt, water, and mint. The method is based on extraction of 10 g of Doogh with 75 mL of methanol and 25 mL of distilled water. An aliquot of 20 µl of Extract after filtration injected to HPLC. This compound was detected using a Grace Smart RP C18 column (100 x 4.6 mm, 3 µm I.D.) and the mobile phase used, was acetonitril: 30mM perchloric acid (35:65) in 5 min. The flow rate was maintained at 0.5 mL/min and the analytical signal was measured at 304 nm.

HPLC-UV İLE DOOGH'DA NATAMİSİN BELİRLENMESİ

Özet

Doogh içinde natamisin belirlenmesi için Yüksek Performans Sıvı Kromatografi yöntemi (HPLC-UV) ile basit ve hızlı bir yöntem, ilk olarak geliştirilmiştir. Doogh İran'a özgü yoğurt, su ve nane ile yapılan serinletici bir içecektir. Yöntem, 10 g Doogh örneğinin 75 mL metanol ve 25 mL saf su ile ekstraksiyonu esasına dayanmaktadır. 20 µL ekstrakt, filtre edildikten sonra HPLC'ye enjekte edilmiştir. Natamisin, Grace Smart RP C18 kolonunda (100 x 4.6 mm, 3 µm I.D.) belirlenmiştir ve mobil faz asetonitril:30 mM perklorik asittir (35:65). Süre 5 dakikadır. Akış hızı 0.5 mL/ dakikadır ve analiz 304 nm'de gerçekleştirilmiştir.

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INTRODUCTION

Natamycin (Pimaricin) (Figure 1), a polyene macrolide antibiotic, is a potent antifungal compound that can effectively prevent the growth of yeasts and inhibit aflatoxin formation in molds (1). Natamycin is widely used in the food industry as a natural food preservative for the prevention of mold contamination of beverages, cheese, fruits and other no sterile foods (i.e., cured meats, sausages) (2, 3). The activity of natamycin against yeasts and molds, but not bacteria, makes it convenient for use in foods that undergo a ripening period after processing. Its low solubility in water and most organic solvents makes it suitable for the surface treatment of foods (4).

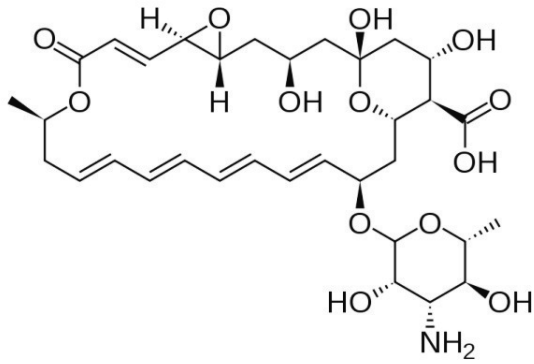

Figure 1. Chemical structure of natamycin.

Natamycin has an empirical formula of $C_{33}H_{47}NO_{13}$ and a molar mass of 665.73. the international Union of Pure and Applied Chemistry (IUPAC) chemical name for natamycin 1*R*, 3*S*, 5*R*, 7*R*, 8*E*, 12*R*, 14*E*, 16*E*, 18*E*, 20*E*, 22*R*, 24*S*, 25*R*, 26*S*)-22-[(3-amino-3,6-dideoxy-D-mannopyranosyl) oxyl]-1, 3, 26-trihydroxy-12-methyl-10-oxo-6, 11, 28-trioxatricyclo [22.3.1.05,7] octacosa-8, 14, 16, 18, 20-pentaene-25-carboxylic acid (5). The primary structure of natamycin is its large lactone ring of 25 C-atoms. The lactone ring is linked to a mycosamine moiety, amino-sugar, by a glycosidic linkage, classifying natamycin as a polyene macrolide antibiotic. In particular, natamycin is a tetraene antibiotic because of its four conjugated double bonds. Natamycin is a white, tasteless and odorless powder (6). Natamycin is extremely insoluble in water. Schaffner and Mechlinski (7) report the solubility of natamycin as 0.0520 mg/mL. In addition, natamycin is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol and various oils. Glacial acetic acid, methyl pyrrolidone, dimethyl formamide, dimethyl sulfoxide, glycerol, and propylene glycol are good solvents for natamycin (8).

The photolysis of natamycin in methanolic solution is very rapid upon light exposure to a xenon lamp with a spectrum similar to that of natural sunlight (9). 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. 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 (10). At high pH values, the lactone is rapidly saponified, forming the biologically inactive natamycoic acid (11). 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 (12). A moderate reduction in biological activity was observed after a typical heat sterilization process (13). Oxidative inactivation of natamycin has also been reported (12, 14). Oxidative inactivation may be prevented by the addition of chlorophyll, ascorbic acid or a number of other antioxidants (15). The LD_{50} of natamycin after oral administration has been determined to be 1.5 g/kg for mice and rats and 0.45 g/kg for guinea pigs. Levinskas et al. determined the LD_{50} as 2.7 - 4.7 g/kg and 1.4 g/kg for rats and rabbits, respectively. The intake of natamycin did not cause any gross lesions on the animals that died (16).

Natamycin is applied to the Doogh either as a liquid suspension or a powder. Doogh is a traditional Iranian fermented milk drink. Apart from Iran, Doogh is exported and consumed in other countries such as Afghanistan, Azerbaijan, Armenia, Iraqi, Syria, Turkey, and Balkans and to less extent in other countries of Middle East and central Asia. The word 'Doogh' has been adopted from Persian term of 'dooshidan' means 'milking'. Traditionally, Doogh was referred to a product obtained from dilution of full fat yogurt

after vigorous agitating in special leather bags, called 'Mishk'. Nowadays, Doogh comprises its specific physical, chemical, physicochemical, microbiological and sensory characteristics that are characterized in Iran National Standard No.2453. In present time, Doogh is a very popular and highly consumed product in 'Iran with a considerable increasing demand for its consumption. It is now known as 'Iran National Drink'. The popularity of Doogh arises from its specific organoleptic characteristics along with its health benefits as a healthy drink based on fermented milk. Some surveys show that Doogh is also potentially acceptable in European countries and a day by day increasing demand is being observed for its export and consumption (17). Many analytical methods are available for natamycin determination. These methods use various techniques for the determination of natamycin in cheese and cheese rind including microbiological and immunochemical procedures (18). Spectrophotometry (19) derivative spectrophotometry (20) and liquid chromatography (20, 21).

Natamycin is non-toxic, non-mutagenic, non-tetratogenic and non-allergenic (22), but based on Iranian National Standard No. 2453 additions any additive is unallowable to Doogh, therefore a simple High-Performance Liquid Chromatographic method with UV detection (HPLC-UV) for the first time to determination of natamycin in Doogh has been developed.

MATERIALS AND METHODS

Experimental Procedures

Reagents

Doogh samples were obtained locally from Mashhad (Khorasan, Iran). Methanol, acetonitril, perchloric acid and other chemical reagents were HPLC grade and supplied by Merck (Darmstadt, Germany). Natamycin powder was obtained from Sigma (St. Louis, USA). Double distilled water was used in all experiments. Stock standard solution was prepared by methanol. Working standard solutions were prepared daily by diluting the stock solution with methanol: water (3:1 v/v). All of the solutions were stored in darkness and refrigerator at 4 °C.

Apparatus

HPLC analyses were performed on a Sykam (Eresing, Germany) HPLC system equipped with an S2100 pump, an S7131 reagent organizer, an S4011 column thermo controller, an S3240/3210 UV_Visible detector and a 3µm GraceSmart RP C18 analytical column (100 x 4.6 mm I.D.). The guard column was ODS-H (20 x 4.6 mm I.D.). Clarity software was used for data management. UV-Visible spectra of natamycin standard solutions and samples were obtained using a Shimadzu UV-1700 Pharma spec. (Tokyo, Japan). Spectrophotometer equipped with a standard 10 mm path length spectrophotometer cell.

Extraction of natamycin

A 10 g portion of Doogh, to the nearest 10 mg, was weighed into a 200 mL conical flask and 75 mL of the methanol was added to the test portion. The contents of the conical flask were stirred for 5 min in a shaking machine. After this, 25 mL of distilled water was added and immediately was placed in the freezer (-20 °C) for precipitation of interfering extractable compounds and was allowed to stand for about 60 min. The cold extract was filtered through a folded filter paper and the first 5 mL of filtrate was discarding. The filtration has to be carried out while the suspension is still cold to avoid dissolution of the fat and consequently turbid filtrates. Extraction was conducted at room temperature in the dark to avoid possible degradation, since natamycin sensitive to light (23, 24).

Chromatographic Method

Linear isocratic elution chromatography has done using solvent system acetonitril: 30 mM perchloric acid (35:65) (25) at 30 °C. The flow rate was kept constant at 0.5 mL/min and 20 µL was injected. The chromatograms were recorded at 304 nm. All analyses were performed at least four times.

Quantitative determination

An external standard curve was constructed using reference standard natamycin to qualify the free natamycin content in all samples. Methanol stock solution containing natamycin 1000 µg/mL was prepared and then diluted to appropriate

concentration ranges with methanol: water (3:1 v/v) for construction of calibration curve. A series of 6 standards of natamycin solutions in methanol: water (3:1 v/v) (from 2.5 to 75 µg/mL) was injected three times to obtain the corresponding calibration curve. The R2 parameter was 0.997. Calibration curve exhibited good linear regression (Figure 2).

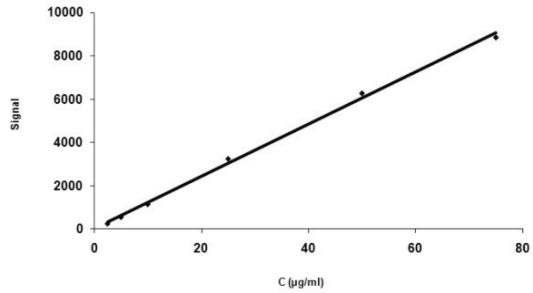

Figure 2. Calibration curve of natamycin.

RESULTS AND DISCUSSION

Doogh was referred to a product obtained from dilution of full fat yogurt after vigorous agitating in special leather bags, therefore the Doogh matrix is a suitable fermentation medium for the lactic bacteria; the antibiotic natamycin is added to avoid this degradation. To determine this drug it must first be extracted from the aqueous matrix. Natamycin is almost insoluble in many solvents. Methanol was used for extraction of natamycin from Doogh samples. Methanol was selected for 4 reasons (25):

1- Natamycin is soluble in methanol

2- Solutions of natamycin in aqueous or pure methanol are stable

3- Methanol can precipitate proteins

4- Methanol has a low level of toxicity

UV Spectra

A 10 µg/mL standard solution of natamycin in methanol: water (3:1 v/v) has three absorption maximum at about 318.2, 304 and 290.6 nm, a shoulder at about 278.8 nm and exhibits minima at about 311.4 and 296 nm (Figure 3). On the basis of observed spectrum, the wavelength 304 nm was selected for the detection and quantification of natamycin.

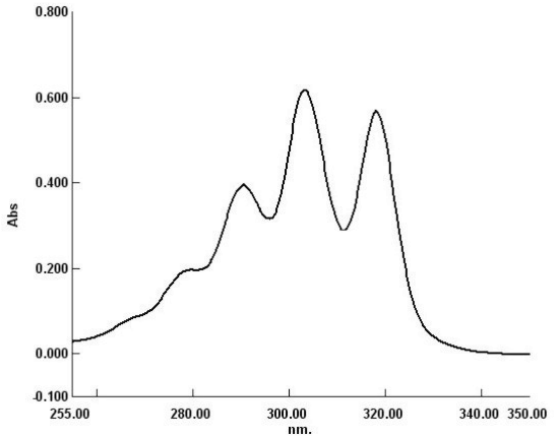

Figure 3. Standard UV spectrum of natamycin (10 µg/mL).

HPLC Analysis

The LC chromatogram of standard solution of natamycin (25 µg/mL) and Doogh sample at its analysis wavelength, 304 nm in the system with acetonitril: 30mM perchloric acid (65:35) as the mobile phase is shown in Figure 4. A good separation is achieved in ca. 5 min. relevant data concerning the analytical system are summarized in Table 1. For natamycin, LOD, LOQ and sensitivity are 1.65, 5.51 and 0.57 respectively (26). Natamycin retention time is 3.78 min. Identification of the analyte in Doogh extract was confirmed by direct comparison of both UV spectra and the retention time of each analyte with those obtained from the authentic standards.

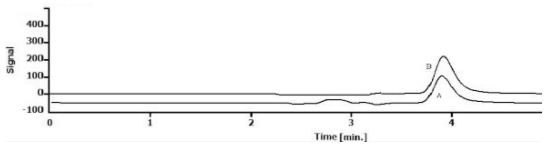

Figure 4. LC chromatogram of standard solution of natamycin (25 µg/mL) (B) and spiked Doogh sample with 1.7 mg natamycin (A).

Accuracy was examined by the determination of the recoveries of the natamycin. The recovery s-tudy was performed by comparing the concentration in the Doogh spiked samples to the respective non-extracted standards (natamycin in solution). The area under the peak of each sample was divided by the area under the curve of the quality control sample and multiplied by 100. The recovery results for three levels are shown in Table 2.

Table 1. Analytical data for the natamycin HPLC system.

Equation	sensitivity	Limit of Detection (µg/mL)	Limit of Quantification (µg/mL)
y=120.3x+32.57	0.57	1.65	5.51

Table 2. Recoveries of natamycin.

	Recovery (%)		
Sample (n=4)	Low concentration (10 mg/kg)	Medium concentration (15 mg/kg)	High concentration (20 mg/kg)
Sample (Doogh)	98.7	101	99.5

CONCLUSION

In conclusion, a novel and simple HPLC method has been developed to quantify natamycin in Doogh. It was successfully applied to the quantification this compound in Doogh. Results indicate that the developed HPLC assay can be readily utilized as a quality control method for natamycin-containing natural products.

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