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Quantitative determination of nystatin in human plasma using LC–MS after inhalative administration in healthy subjects

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Abstract

The antifungal polyene antibiotics nystatin was tested in a clinical trial to describe pharmacokinetics and safety after repeated administration of Nystatin "Lederle" sterile powder in healthy volunteers. To monitor the nystatin concentration–time profile in plasma we developed a sensitive method in the range of 1–100 ng/ml based on liquid chromatography coupled with tandem mass spectrometry. The target substance was separated from the biological matrix on C_{18} solid-phase extraction cartridges with methanol. The Chromatography was performed isocratically using a reversed phase Caltrex Resorcinearene column. The mobile phase consisted of 5 mM ammonium formate buffer and acetonitrile (40:60, v/v). The mass spectrometer works with electrospray ionization in its positive selected ion monitoring (SIM) mode using the respective MH^+ ions, *m/z* 926.6 for nystatin and *m/z* 924.4 for amphotericin B as internal standard. The method validation was performed according to the demands and international criteria for validation of bioanalytical methods and was successfully applied to the quantification of nystatin in human plasma in the pharmacokinetic trial.

Keywords: Nystatin; Inhaled aerosol; Drug assay; LC–MS

1. Introduction

The macrolide antibiotics nystatin was isolated from the actinomycete species *Streptomyces noursei* in 1950 and launched into antifungal therapy just five years later [1]. Nystatin is an efficient antimicrobial agent against a broad spectrum of saprophytic and pathogenic fungi [2,3]. Its current clinical use, however, is limited to topic applications on the skin and mucous membranes because of its low tolerability after parenteral administration [4]. Nystatin and the structurally related amphotericin B are lactones consisting of a hydroxylated polyene macrolide backbone connected by a glycosidic bond with mycosamine (Fig. 1). Both drugs are not absorbed from the gastrointestinal tract, skin, or vagina [3,5]. Nystatin is on the market in several pharmaceutical formulations for oral treatment of gastro-intestinal mycoses and for many other topical applications in dermatology, gynecology or otology [3]. Nystatin "Lederle" sterile powder (ICN Pharmaceuticals, Frankfurt/Main, Germany) is on the marked for aerosol therapy in pulmology. Contrary to inhaled amphotericin B, there is to our knowledge no information on disposition and safety of nystatin after pulmonary administration [6]. So far, a few HPLC methods are available to measure nystatin plasma concentration–time profiles after parenteral administration of high doses in animals [7–9]. The limits of quantification of these methods (above 50 ng/ml) are not sufficient to quantify the drug in human plasma after pulmonary administration of therapeutic doses. Therefore, we described in this paper the development and validation of a novel LC–MS method for nystatin measure plasma concentrations up to the detection limit of 1 ng/ml in a pharmacokinetic study after chronic inhalation of therapeutic doses of the drug in man.

2. Methods

2.1. Quantitative assay of nystatin in plasma

Nystatin was provided by ICN Phamaceuticals (Frankfurt/Main, Germany) as a yellow crystalline powder. Because

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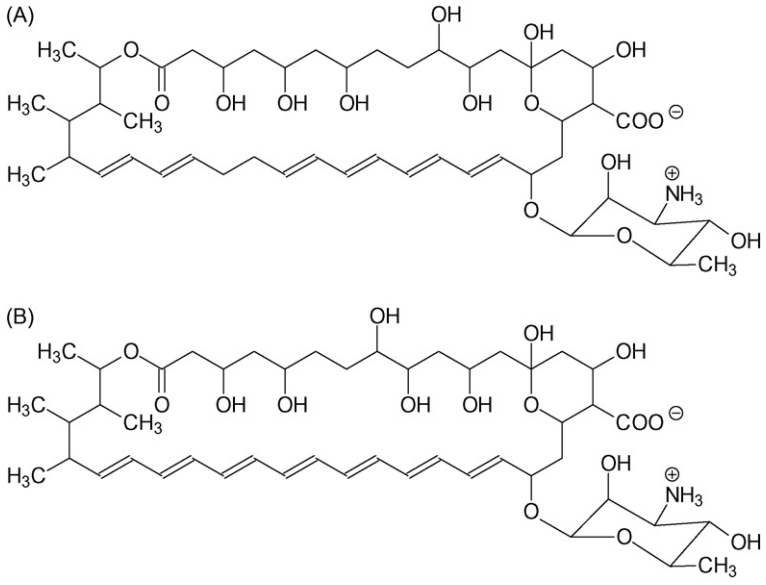

Fig. 1. Chemical structure of nystatin (A) and amphotericin B (B).

no stable isotope-labeled nystatin was available, the structurally related amphotericin B was used as internal standard (Sigma–Aldrich, Taufkirchen, Germany). Amphotericin B differs from nystatin by transition of the OH-group and an additional double bond (Fig. 1). Ammonium formate and formic acid were purchased from Merck (Darmstadt, Germany), acetonitrile and methanol (LC–MS Chromasolv®) from Baker (Gross, Gerau, Germany). Deionized water (conductance: ≤0.055 μS/cm, pH 5.0–6.0) was generated using the SG system RF 40 EZ (Hamburg, Germany).

2.1.1. Sample preparation

To 1.0 ml plasma (sample from the clinical study, calibrator or quality control sample), 25 μl of the internal standard solution (1.0 μg/ml amphotericin B) and 1.0 ml water were added. One milliliter of this solution was extracted with C18 SPE Bond Elut C_{18} cartridges (Varian, Darmstadt, Germany) using the Gilson Aspec XL apparatus (Abimed, Langenfeld, Germany). The cartridges had been preconditioned with 1.0 ml methanol. After extraction, cartridges were washed with 1.0 ml water and dried with compressed air. Then, the analytes were eluted twice with 0.4 ml methanol. The combined elutes were evaporated to dryness at 30 °C under a gentle air stream and dissolved in 50 μl mobile phase. Aliquots of 10 μl were injected into the LC–MS system.

2.1.2. Chromatography and mass spectrometry

The LC–MS/MS system consisted of the API 2000 triple quadrupole mass spectrometer equipped with the electrospray ionization (ESI) source TurboIonSpray™ (Applied Biosystems, Darmstadt, Germany), a binary pump with integrated degasser (Hewlett-Packard Series 1100, Waldbronn, Germany), the column oven L-5025 (Merck-Hitachi, Darmstadt, Germany) set at 25 °C, an autosampler equipped with the Peltier cooling system set to 15 °C (Perkin Elmer Series 200, Darmstadt, Germany), and the Caltrex Resorcinearene® column (125 mm × 2 mm, particle size 5 μm; Caltrex, Greifswald, Germany). Caltrex Resorcinearene® is a new separation phase consisting of condensed alkyl substituted diphenols which are bound to silicagel carrier material.

The chromatography was performed isocratically using 5 mM formate buffer/acetonitril (40:60, v/v) as mobile phase with a flow rate of 0.3 ml/min.

The mass spectrometer operated in the single ion monitoring (SIM) mode by TurboIonSpray™ ionization. Peak areas of the respective protonated molecules were measured (nystatin, *m/z* 926.6; amphotericin, *m/z* 924.4). Details of the optimized TIS and MS parameters are given in Table 1.

The chromatograms were evaluated with the internal standard method using peak-area ratios for calculation of the calibration function and linear regression analysis weighted by $1/x$ (x = concentration). For data acquisition and statistical evaluation, the device-specific software Analyst 1.2 (Applied Biosystems, Darmstadt, Germany) and the program package Microsoft® Office Excel 2003 (Microsoft Cooperation, Redmond, USA), respectively, were used.

2.1.3. Method validation

Validation of the quantitative assay was performed according to the accepted international recommendations [10–12].

Table 1
Optimized TIS and MS parameters for the determination of nystatin

ESI parameters	MS parameters
Nebulizer gas: 50 psi	Declustering potential: 20 V
Auxiliary gas: 85 psi	Focusing potential: 400 V
Temperature: 200 °C	Entrance potential: 10 V
Curtain gas: 20 psi	Capillary voltage: 5000 V

Nitrogen was used as nebulizer, auxiliary and curtain gas (1 psi = 6894.8 Pa).