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Short communication

HPLC method for the determination of nystatin in saliva for application in clinical studies

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Abstract

An isocratic high-performance liquid chromatographic method was developed, optimized and validated for the determination of nystatin in human saliva (UV and fluorescence detection). A reversed-phase Luna™ C18 column (25 °C), with a mobile phase of MeOH, H_2O, and DMF (70:20:10, v/v/v), and a flow-rate of 0.8 ml/min were used. The elution time for nystatin was 5.8 ± 0.2 min. Calibration curves in human saliva were linear from 0.78 to 50 μg/ml. Limits of quantification were 0.78 μg/ml and 0.75 μg/ml for UV and fluorescence detection, respectively. The accuracy and precision values of intra- and inter-day variation studies were within acceptable limits, according to FDA guidelines. The described method has proved to be useful to give accurate measurements of nystatin in real samples.

Keywords: Nystatin; Human saliva; HPLC; Validation

1. Introduction

Nystatin is a macrocyclic lactone consisting of a hydroxylated tetraene diene backbone and a mycosamine residue (Fig. 1). This drug primarily acts by binding to ergosterol, the principal sterol on fungal cell membranes, resulting in the formation of transmembrane channels [1]. Nystatin exerts both a fungistatic and fungicidal action against *Candida Albicans*. For the treatment of oral candidiasis, this drug is administrated in either a suspension or gel dosage forms (100,000 IU) taken 4–5 times daily [2]. Currently, available therapies do not always successfully relieve oral candidiosis, due principally to the fast diminution of nystatin salivary levels as a consequence of the salivary turnover (0.5–2.0 l/day) and swallowing. In this way, the drug and occasionally the formulation are removed from the buccal cavity.

Therefore, a mucoadhesive formulation, which prolongs the residence time and is able to control the drug release toward oral cavity, is expected to overcome these problems. In this sense, different release systems (gels, tablets and films) have been developed [3,4].

The evaluation of the biopharmaceutical performance of these novel formulations requires the development of adequate analytical methodologies. However, the physico-chemical properties of nystatin [5], particularly its low solubility in solvents currently used in HPLC and UV determinations, makes quite difficult its determination in biological fluids.

Although, different procedures have been described in the literature regarding the determination of nystatin in urine, blood, tissues and saliva [1,6], this is the first report on the development and validation of a specific HPLC method for its analysis in saliva, because the early paper refers to an absolutely outdated microbiological assay [7]. Comparing to the Groll et al. method for the determination of nystatin in plasma and tissues [1], our method uses a simple mobile phase composition, easy to prepare. The low flow rate, mobile phase consumption and short retention times prove cost-effective.

In a previous work [8], we reported the design of a mucoadhesive tablet able to maintain enough nystatin concentration in saliva for approximately 4 h. The *in vivo* evaluation of the performance of this sustained release tablet required the development of an appropriate HPLC analytical methodology that is reported here. In this context, we developed a HPLC method for the quantification of nystatin in saliva which was based upon ultraviolet (UV) and fluorescence detection. This method has been fully validated following present guidelines [9,10] and a stability study

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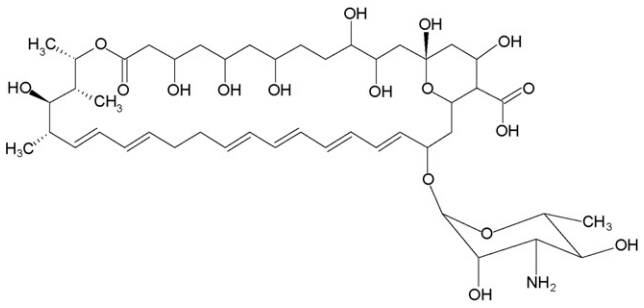

Fig. 1. Chemical structure of nystatin.

was also included. The assay appeared to be very robust and sufficiently sensitive for drug level monitoring.

2. Materials and methods

2.1. Materials

Bulk nystatin (N) was supplied by Unifarma S.A. (Buenos Aires, Argentina). Pooled normal saliva was collected from healthy untreated volunteers. Methanol (MeOH), acetonitrile (AcN) and dimethylformamide (DMF) were all HPLC-grade (Sintorgan, Argentina). Milli-Q water was used in all experiments.

2.2. Chromatographic system

The chromatographic system (Brezee system) consisted of a Waters 1525 pump, a Waters 717 plus autosampler, a Waters 1500 series column heater, a Waters 2475 multi λ fluorescence detector (λ_{ex} 290 nm, λ_{em} 410 nm) and a Waters 2996 photo array detector (PDA) (Waters Corp., Milford, USA). The wavelength of this detector was set at 305.6 nm. Data acquisition was performed by the Empower Software data registration™. The analytical column was a reversed-phase Luna™ C_{18} (250×4.6 mm i.d., 10 μm particle size, Phenomenex™) maintained in the column oven at 25 °C and protected by a SecurityGuard™ precolumn. The mobile phase consisted of MeOH:H_2O:DMF (70:20:10, v/v/v). Elution was performed isocratically at 25 °C at a flow-rate of 0.8 ml/min. The mobile phase was filtered through a 0.45 μm Millipore™ Durapore filter and degassed by vacuum prior to use.

2.3. Preparation of standard solutions

A stock standard solution of nystatin was prepared by weighing out an appropriate amount of bulk nystatin (BN) and then mixing it with 10 ml of saliva in a polypropylene centrifuge tube. This solution was stored at 37 °C and protected from light for 90 min. At 10 min periods the sample was vortexed for 1 min. After this the sample was centrifuged (15 min 5000 RPM), the supernatant was collected and transferred to a glass container and stored at 0 °C (until calibration standards were prepared). The precipitate was resuspended in MeOH and then assayed spectrophotometrically in order to determine how much nystatin (P) was not soluble in saliva. The amount of soluble nystatin (S) was calculated according to following equation:

$$(BN) - (P) = (S) \quad (1)$$

2.4. Sample preparation

Calibration standards and quality controls (QC) were prepared by diluting different aliquots of the collected supernatant with drug-free saliva until a volume of 600 μl was obtained. These samples were vortex-mixed for 15 s, and a deproteinizing solution of MeOH–AcN (1:1, v/v) was added to each one. Then, the samples were vortex-mixed every 10 min during 30 min, before being immediately centrifuged for 10 min at 5000 × *g*, filtered (Durapore filter), transferred into autosampler vials and injected (50 μl) into the HPLC system.

2.5. Validation parameters

2.5.1. Calibration curves

All standards were prepared and analyzed as follows:

Duplicate six, seven or twelve-points calibration curves were analyzed on each of 2 days. The calibration curves were obtained by linear regression analysis of the sum of the peak-areas of the two major peaks of nystatin versus its concentrations. The determination coefficients (r^2), and relative standard deviations (R.S.D.%) of the response factors of each standard were calculated each day. Also, the linearity was assessed by plotting the interpolated concentrations against their nominal values.

2.5.2. Lower limit of quantification

The lower limit of quantification is defined as the concentration of the lowest standard in the analytical run which is quantified with a deviation of the actual concentration and a coefficient of variation of precision of less than 20%.

2.5.3. Accuracy and precision

The accuracy and precision of the method were determined by replicate analysis of QC samples at three (intra-day) or five (inter-day) concentration levels in the high, medium and low concentration range of the calibration curves.

2.5.4. Selectivity

Selectivity was determined using blank samples: pool of saliva from 10 donors and a deproteinizing solution of MeOH–AcN (1:1) without addition of nystatin.

2.5.5. Stability

The stability of nystatin was tested with calibration standards that were stored in a freezer for 5 weeks. The samples were analyzed immediately after the storage period.

Analytes stability was also determined after freeze and thaw cycles. Samples of low and high concentration levels underwent three freeze and thaw cycles and were analyzed after each cycle. The samples were frozen for 24 h each time and thawed at room temperature.

Stability in the autosampler was also evaluated. QC samples at the two concentration levels were stored at room temperature and assessed after 6 h, which was the run time for each batch.

3. Results and discussion

3.1. Optimization of conditions and chromatograms

Nystatin is almost insoluble in aqueous solutions, whereas it is freely soluble in organic solvents like methanol, acetonitrile and dimethylformamide. During the development stage, the use of methanol, acetonitrile and water as the mobile phase resulted in asymmetric peaks with tailing factors over 2. The addition of dimethylformamide to the mobile phase instead of acetonitrile yielded a drastic reduction of peak tailing. At the reported mobile phase composition, the tailing factors (1.2) were within the acceptable limit resulting in good peak symmetry and resolution. Increasing the flow rate to 1.0 ml/min and 1.2 ml/min resulted in poor resolution between the drug and the saliva components, whereas the decrease of the flow rate to 0.6 ml/min gave a drug retention time greater than 10 min. Hence, the mobile phase flow rate was set at 0.8 ml/min. In the optimized conditions, acceptable retention times of around 5.8 min and 6.8 min were determined for the two peaks of nystatin using a C18 analytical column preceded by a guard column. A representative chromatogram from a saliva sample preparation containing nystatin is shown in Fig. 2. According to published results [1] we assigned the major peak to nystatin and the minor peak to its isomer, which is formed in biological fluids. Also, we determined that this minor peak appeared at concentrations above 5.0 μg/ml.

The selectivity of the method was also tested by observing potential interferences with nystatin peak arising from the saliva components or the deproteinizing solution. No interfering peaks were observed at the retention times of nystatin. A chromatogram corresponding to a blank saliva sample is shown in Fig. 2b.

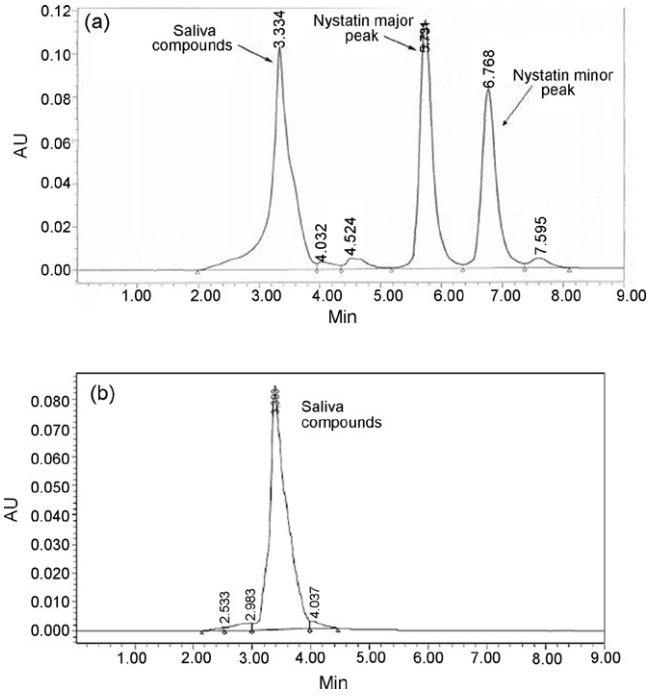

Fig. 2. Representative HPLC-UV chromatograms from a saliva sample preparation containing nystatin (a) and a blank saliva sample (b).

3.2. Linearity

The calibration graphs were obtained by plotting the sum of nystatin peak areas against the corresponding concentrations. A concentration range of 4.2–50 μg/ml was used for curves 1, 2, 5 and 6, while a wider range of concentrations was used for curves 3, 4 and 7 in which the lower limit was extended up to 0.75 μg/ml.

The upper and lower parts of Table 1 report respectively, UV and fluorescence detectors results. Calibration curves were linear over the concentration range studied, with determination coefficients being greater than 0.991 for all curves.

Table 1
Results of regression analysis of the linearity data of nystatin

$y = bx + a$	c_1	c_2	c_3	c_4	Mean
UV detector					
a (intercept)	3.1×10^4	2.7×10^4	-4.1×10^4	-6.3×10^4	-1.2×10^4
b (slope)	6.5×10^7	6.6×10^7	6.6×10^7	6.8×10^7	6.6×10^7
r^2 (determination coefficient)	0.992	0.997	0.999	0.998	0.997
n (number of points)	6	7	12	12	
Response factor R.S.D.%	4.5	4.9	3.7	6.4	4.9

$y = bx + a$	c_5	c_6	c_7	Mean
Fluorescence detector				
a (intercept)	2.8×10^8	-1.3×10^7	7.3×10^7	1.1×10^8
b (slope)	6.3×10^{10}	6.0×10^{10}	6.3×10^{10}	6.2×10^{10}
r^2 (determination coefficient)	0.992	0.991	0.994	0.992
n (number of points)	6	6	12	
Response factor R.S.D.%	5.2	5.4	6.0	5.5

Response factor = peak area/standard concentration.

Table 2
Precision and accuracy of the HPLC assay for nystatin in saliva

Nominal concentration (μg/ml)	Calculated concentration (mean ± S.D.) (μg/ml)	Precision R.S.D.%	Accuracy (%)	*n*
Day 1				
UV detector				
4.1	3.6 ± 0.2	5.6	88	5
25.1	23.7 ± 0.1	3.9	95	5
50.2	49.9 ± 0.7	5.8	99	5
Fluorescence detector				
4.2	4.2 ± 0.4	12.1	100	5
25.1	24 ± 2	3.4	96	5
50.2	49 ± 2	8.8	98	5
Day 2				
UV detector				
0.78	0.79 ± 0.05	6.3	101	6
2.35	2.1 ± 0.2	3.6	89	6
3.91	3.4 ± 0.3	1.4	87	6
23.4	23 ± 1	1.0	98	6
46.9	47 ± 5	5.0	100	6
Fluorescence detector				
0.75	0.6 ± 0.1	11.2	85	6
2.46	2.1 ± 0.1	3.4	90	6
4.10	3.6 ± 0.3	7.7	88	6
24.6	24 ± 2	12.1	98	6
49.2	50 ± 2	8.9	102	6

S.D.: standard deviation; *n*: number of replicates.

The linearity of the curves was also assessed by the response factors, since these values represent the slope of a calibration curve at each point. The calculated values indicated a linear behavior, for all curves. The R.S.D.% of the response factors presented in Table 1 shows an acceptable correlation between peak area and concentration, since all the values obtained were <6.5%.

In subsequent evaluations that were conducted to check the linearity, the regression equations obtained between interpolated and nominal concentrations gave slopes very close to 1 and intercepts near to 0 (data not shown).

3.3. Precision, accuracy and lower limit of quantification (LOQ)

Precision and accuracy calculated for the QC samples during the intra- and inter-day run are given in Table 2.

The accuracy of the assay method, defined as the closeness of the agreement between the mean of the measured values and the true value, to be acceptable, should be within ±15% of the actual value at all concentrations. The accuracy values in intra- and inter-day variation studies at low, medium and high concentrations for nystatin in human saliva were within acceptable limits.

The precision of a method, expressed as the relative standard deviation percentage (R.S.D.%) of replicates, to be acceptable, should lay within ±15% at all concentrations. In this work, precision of the method was tested by both intra- and inter-day repeatabilities. In all cases R.S.D.% were lower than 15% suggesting an adequate repeatability of the assay method. Using the UV detector, precision was better than 6.3%, whereas with the fluorescence detector slightly higher R.S.D.% were observed, but were still less than 15% as recommended by the FDA guidelines.

The limit of quantification (LOQ) was taken in this work as the lowest concentration standard affording accuracy and precision ≤20%. Even though the LOQ values obtained were around 0.7 μg/ml and similar for both methods, precision and accuracy have resulted slightly better for UV detection, in spite of being fluorescence detection considered more sensible.

Fluorescence detection was used concurrently with absorbance detection to determine if greater selectivity and/or sensitivity could be obtained via fluorescence versus UV detection of nystatin. The results obtained indicated that the validation parameters were practically the same using both UV and fluorescence detection.

3.4. Sample stability

In most cases the analysis of the biological samples for drug testing is not performed immediately after sample collection. Therefore, it is important to test if the drug is stable during study time [11,12].

Table 3 shows the results for the long-term, the freeze–thaw, and the short-term stability studies. Although samples stability in freeze/thaw studies was analyzed after each of the three cycles, Table 3 only shows the obtained results at the end of the third cycle. Also, the effect of storage conditions on samples stability was assayed only after 5 weeks.

All results were satisfactory since no value was smaller than 85% of the initial concentration, as FDA recommends [10].

Table 3
Results of the stability study of Nystatin under different conditions

	Low QC ($n=3$)		High QC ($n=3$)	
	Recovery (%)	R.S.D.%	Recovery (%)	R.S.D.%
−20 °C (5 weeks)	89.5	3.4	92.6	2.6
Freeze/thaw	94.6	2.8	93.5	1.7
RT (6 h)	95.3	3.2	96.8	2.1

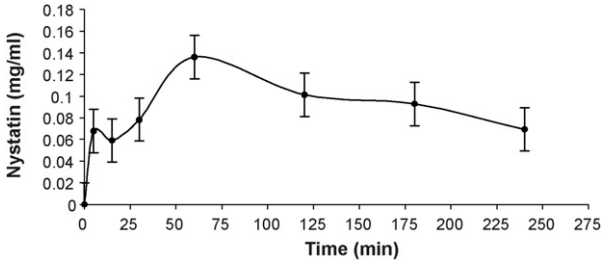

Fig. 3. Concentration–time profile of nystatin in saliva.

3.5. Clinical applications

The saliva samples from four healthy volunteers, to whom were administered mucoadhesive tablets containing 10 mg of nystatin, were assayed with the validated method described above. The mean concentration–time curve is shown in Fig. 3.

3.6. Conclusions

This paper describes a rapid and reproducible HPLC method which enables the determination of nystatin in saliva. The typical assay time is about 10 min. The relatively short retention times of the drug in our technique allows the analysis of a large number of samples over a short time. In conclusion, the HPLC method developed using both UV and fluorescence detection shows a good sensitivity and selectivity and is suitable for reliable determination of nystatin in saliva. The HPLC assay method presented here has been successfully applied to the evaluation of the biopharmaceutical performance of the novel mucoadhesive formulation.

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