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High-performance liquid chromatographic determination of liposomal nystatin in plasma and tissues for pharmacokinetic and tissue distribution studies

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Abstract

A reliable reversed-phase high-performance liquid chromatographic method was developed for the determination of liposomal nystatin in plasma. Nystatin is extracted by 1:2 (v/v) liquid–liquid extraction with methanol. Separation is achieved by HPLC after direct injection on a μBondapak™ C_{18} analytical column with a mobile phase composed of 10 m*M* sodium phosphate, 1 m*M* EDTA, 30% methanol and 30% acetonitrile adjusted to pH 6. Detection is by ultraviolet absorbance at 305 nm. Quantitation is based on the sum of the peak area concentration of the two major isomers of nystatin, which elute at 7.5–8.5 and 9.5–10.5 min. The assay was linear over the concentration range of 0.05 to 50 μg/ml. The lower limit of quantitation was 0.05 μg/ml, sufficient for investigating the plasma pharmacokinetics of liposomal nystatin in preclinical studies. Accuracies and intra- and inter-day precision showed good reproducibility. With minor modifications, this method also was used for assaying nystatin in various non-plasma body fluids and tissues.

Keywords: Nystatin

1. Introduction

Nystatin ($C_{47}H_{75}NO_{17}$; mol.wt. 926.13), discovered as the first antifungal polyene antibiotic in the early 1950s [1], is a macrocyclic lactone consisting of a hydroxylated tetraene diene backbone and a mycosamine residue (Fig. 1) [2]. In common with other polyenes such as amphotericin B, nystatin primarily acts by binding to ergosterol, the principal sterol of fungal cell membranes, resulting in the formation of transmembrane channels and ultimately, death of the fungal cell [3]. Similar to amphotericin B, nystatin has potent and broad-spectrum antifungal activity in vitro. However, early problems with solubilization and toxicity after parenteral administration precluded the compound's use for systemic treatment [4].

More recently, nystatin has been incorporated into

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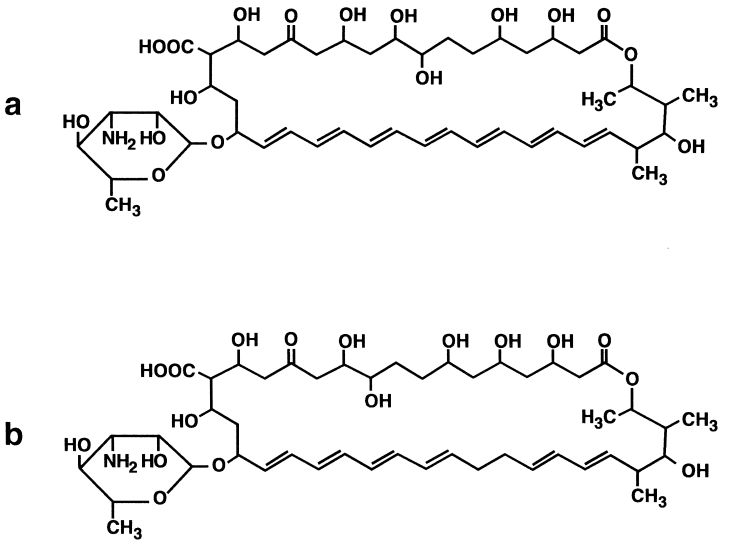

Fig. 1. Structural formula of amphotericin B (a, top) and nystatin (b, bottom). Both compounds share a macrocyclic C-41 polyene lactone linked glycosidically to the pyranose-form of the amino sugar mycosamine. The aglycon part of the molecule contains a diene and tetraene chromophore in nystatin, and a heptaene chromophore in amphotericin B.

a multilamellar liposome preparation consisting of dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) in a 7:3 molar ratio and a particle diameter of 0.1 to 3 μm. The multilamellar liposome formulation has reduced toxicity to mammalian cells but preserved in vitro antifungal activity [5–7] and it has demonstrated encouraging activity in animal models of invasive fungal infections [8–10]. It was well tolerated at dosages of up to 8 mg/kg/day in a phase I clinical trial [11] and is currently undergoing phase II and III clinical trials in patients with proven or suspected invasive fungal infections.

In order to characterize the disposition of multilamellar liposomal nystatin in bloodstream and tissues, a method for the quantitation of this new antifungal compound was needed. Therefore, a sensitive and specific method was developed which uses simple liquid–liquid extraction and UV detection. The validation of this method for the extraction of the parent drug from rabbit plasma and various body fluids and tissues is described in this paper and an example of its application to pharmacokinetic studies is given.

2. Experimental

2.1. Materials

Bulk nystatin (purity: 95% nystatin A1) was supplied from Gist Procades (Wilmington, DE, USA). Liposomal nystatin (Nyotran™; 50 mg vials; 50 mg 95% pure nystatin A1 incorporated into a mixture of 350 mg DMPC and 150 mg DMPG) was provided as lyophylized powder by Aronex Pharmaceuticals, The Woodlands, TX, USA and reconstituted according to the manufacturers recommendations. Pooled normal rabbit serum was obtained from Gibco Laboratories (Grand Islands, NY, USA), cerebrospinal fluid (CSF) standards from Instrumentation Laboratories (Lexington, MA, USA), Hank's Balanced Salt Solution (without calcium, magnesium, and phenol red) from Mediatech (Herndon, VA, USA), and pooled level two quality controls for therapeutic drug monitoring from Dade International (Miami, FL, USA). Normal rabbit tissues, bone marrow, bile, and urine were obtained from untreated control rabbits. Methanol and acetonitrile were of HPLC-quality (Fisher Scientific, Fair Lawn, NY,

USA). De-ionized water was processed through an Ultrapure water purification system (Hydro Service & Supplies, Research Triangle Park, NC, USA). Fourteen millilitre polypropylene tubes were obtained from Becton Dickinson (Lincoln Park, NJ, USA), 1.5 ml polypropylene tubes from Robbins Scientific (Sunnyvale, CA, USA), and 0.22 μm Durapore filter tubes from Millipore (Bedford, MA, USA). HPLC injection vials and inserts were from Sun International (Wilmington, NC, USA) and Thomson Instruments (Chantilly, VA, USA) respectively. Ethylenediamine-tetraacetic acid (EDTA) and monobasic sodium phosphate was purchased from Fisher Scientific, phosphate buffered saline pH 7.4 from Quality Biological (Gaithersburg, MD, USA) and heparin sodium USP was obtained from Fujisawa, Inc. (Deerfield, Ill., USA). Tissue micro wipes were obtained from Scott Paper Company (Philadelphia, PA, USA). For homogenization of tissues, a Tizzumizer with a 10 N head was used (Tekmar, Cincinnati, OH, USA).

2.2. Chromatographic systems

The chromatographic system consisted of a Waters 515 HPLC pump, a Waters 717 plus autosampler equipped with a cooling device, and a Waters 486 Absorbance UV detector (Waters Corp., Milford, MA, USA). The wavelength of this detector was set to 305 nm with a response time of 1 sec; the range of detection was 0.001 AUFS. Data acquisition was performed using a CR501 Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). The analytical column was a reverse phase μBondapak™ C_{18} (300×3.9 mm I.D., 100 Å, 10 μm particle size; Waters Corp.) maintained in a column oven (Timberline Instruments, Boulder, CO, USA) and protected by a NewGuard RP-18 precolumn (15×3.2 mm I.D., 5 μm particle size; Perkin Elmer, Norwalk, CT, USA). The mobile phase consisted of 10 m*M* sodium phosphate, 1 m*M* EDTA, 30% HPLC-grade methanol and 30% HPLC-grade acetonitrile, pH adjusted to 6.0 with 85% phosphoric acid. Elution was performed isocratically at 30°C at a flow-rate of 2.0 ml/min. The mobile phase was filtered through 0.2 μm nylon membrane filters (Nalge Company, Rochester, NY, USA) and degassed by vacuum sonication prior to use.

2.3. Preparation of standard solutions

A stock standard solution of nystatin (500 μg/ml) was prepared by weighing out the appropriate amount of bulk nystatin and dissolving it by ultrasonication in methanol; the factor to correct purity was 0.95. Further stock solutions (50, 5, and 0.5 μg/ml) were made by diluting the initial stock standard solution with methanol. Separate stock standard solutions were prepared for standard curves and quality control samples, respectively. The stock solutions were protected from light and stored at −20°C. These solutions were chemically stable for at least 2 months.

2.4. Standard curves and quality control samples

Two seven-point calibration curves, ranging from 0.05 to 1.5 μg/ml (low range) and 1.5 to 50 μg/ml (high range) were used for quantitation of drug in plasma. Quantitation was based on the peak area-concentration response of the calibration curves. Calibration standards were prepared by adding 10–50 μl of nystatin stock solutions to 990–950 μl blank rabbit plasma to a total volume of 1000 μl.

Quality control samples for both low and high range calibration curves were prepared in similar fashion from a separate stock standard to determine the precision and accuracy of the assay and to evaluate the stability of samples under various conditions. Over curve controls were also prepared to evaluate parallelism when specimens required dilution for analysis. Quality control pools were aliquotted and stored at −80°C.

Seven-point calibration curves and two-point quality control samples for matrices other than plasma were similarly prepared by adding known amounts of stock solution to either normal rabbit serum (choroid), normal rabbit bile or urine, commercially available CSF standards, Hank's Balanced Salt Solution (for vitreous and aqueous humor), and normal rabbit tissues 1:2 (w/w) homogenized in methanol. Calibration curves ranged from 0.05 to 1.5 μg/ml (choroid; CSF, vitreous and aqueous humor) and

from 0.05 to 20 for solid tissues and remaining body fluids.

2.5. Sample preparation

Extraction of drug from plasma and body fluids involved addition of 800 μl HPLC-grade methanol to 400 μl of sample (2:1, v/v) and incubation at 4°C for 30 min in 1.5 ml polypropylene tubes. After centrifugation (10 min at 2000 *g*), the supernatant of each tube was transferred to a fresh tube and centrifuged a second time (4 min at 10 000 *g*). 400 μl of the resulting supernatant was then loaded onto a 0.22 μm Durapore filter tube and centrifuged a third time at 4000 *g* for 4 min. The final product was transferred into ambered glass mini-injection vials. An aliquot of 200 μl was injected onto the analytical column. The three-step sample preparation procedure was employed a priori to ensure optimal purity of the material injected onto the analytical column; however, simple precipitation/centrifugation may be equally suitable and less expensive.

Solid tissues were thawed and aliquots of approximately 1 g were weighed out for each sample. The specimens were rinsed with phosphate buffered saline pH 7.4; remaining buffer solution on the tissue surface was blotted with micro tissue wipes. Specimens were then re-weighed, cut in small pieces and homogenized with ice-cold HPLC-grade methanol (1:2, w/w). Tissues were homogenized to completeness in a tissumizer with a 10 N head several times for short intervals with the sample being placed in an ice bucket to avoid heating. Calibration standards and quality control samples for drug analysis in tissues including bone marrow were prepared by homogenizing normal tissue in ice-cold HPLC-grade methanol (1:2, w/w) and adding known amounts of compound. Homogenized samples were incubated for 30 min at 4°C, and centrifuged three times as described above. The final supernatant was pipetted into ambered glass mini injection vials and an aliquot of 200 μl was injected onto the analytical column. Drug concentrations in tissues were calculated to 1 g of tissue. Blank samples of all matrices also were extracted to ensure the absence of endogenous interfering peaks.

2.6. Validation

The method was fully validated for extraction of nystatin from rabbit plasma according to published guidelines [12]. Duplicate calibration curves were analyzed on each of three days. Blank plasma and six quality controls at each concentration were analyzed with each calibration curve. The calibration curves were obtained by least-squares linear regression analysis of the sum of the peak-areas of the two major peaks of nystatin versus the concentration of the compound. The equations of the calibration curves were then used to calculate the concentration of nystatin in samples and controls from the sum of the corresponding peak-areas.

3. Results and discussion

3.1. Separation

Representative chromatograms of blank rabbit plasma, and pooled rabbit plasma spiked with bulk nystatin to 1.25 and 5 μg/ml are depicted in Fig. 2; Fig. 3 shows the chromatograms from plasma from a rabbit receiving 4 mg/kg/day of liposomal nystatin immediately before and at 6 and 1 h after dosing. The mean retention times of the two major peaks of nystatin selected for quantitation in rabbit as well as human plasma were approximately 7.5–8.5 and 9.5–10.5 min. Of note, there were no differences in the appearance of the chromatograms between rabbit and human plasma.

In plasma samples containing high concentrations of either bulk or liposomal nystatin, at least seven different peaks could be identified (Fig. 4). Preliminary results of mass spectroscopy on rat plasma spiked with liposomal nystatin indicate that both major peaks have the appropriate mass for the A1-isomer of nystatin [data on file, Aronex Pharmaceuticals]. These two peaks might represent the Z/E isomers of the diene; however, their exact chemical substrates, whether they confer different pharmacological activities, and whether they occur as a result of the analytical method only or whether they also occur in vivo are unresolved questions subject to ongoing investigation. The remaining peaks might

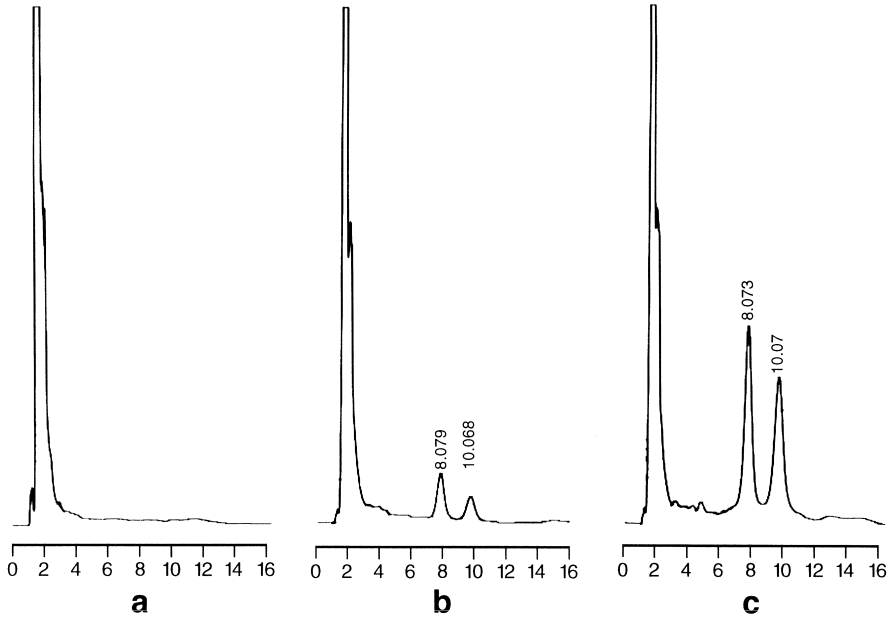

Fig. 2. Chromatograms of extracted drug-free pooled normal rabbit plasma (a) and extracted rabbit plasma spiked with bulk nystatin to a concentration of 1.25 μg/ml (b) and 5.0 μg/ml (c). The sum of the peak areas of the two major peaks of nystatin, which eluted at 7.5–8.5 and 9.5–10.5 min, was used for quantitation.

represent hemiketal forms, which can arise from the formation of an oxygen bridge between carbon atom 13 and 17 of the hydroxy-ketone moiety of nystatin [13,14] and minor quantities of other very closely related compounds, a finding that is common for the class of biosynthetically derived antifungal polyenes.

3.2. Specificity

To assess the validity of using bulk nystatin as reference standard for the analysis of samples containing nystatin encapsulated into the liposomal formulation, normal rabbit plasma ($n=3$) was spiked with liposomal nystatin to a final concentration of 5, 10, and 20 μg/ml. Plasma samples were extracted, submitted to assay and quantified using a high range standard curve simultaneously prepared from bulk nystatin. Accuracies at the three concentrations were +4%, −8%, and −3% of the expected value, and precision as assessed by the coefficient of variation of the mean was 10.90%, 2.79%, and 2.63%, respectively.

There was no interference from endogenous plasma components in both rabbit and human plasma. In addition, there was no chromatographic interference by various other commonly prescribed medications, as assessed by extraction and analysis of blank and spiked human plasma-derived commercially available quality controls for therapeutic drug monitoring. Medications exhibiting no chromatographic interference are listed in Table 1.

3.3. Linearity, precision, and accuracy

Calibration curve data and parameters for nystatin are given in Tables 2 and 3. Calibration curves of nystatin in plasma were linear over the concentration range of 0.05 –50 μg/ml, with r^2-values of greater than 0.992 for all curves. The two-curves calibration system was utilized because the exact range of concentrations of nystatin in samples from pharmacokinetic studies was not known initially, and preliminary data indicated that a large range of concentrations was to be expected. A single calibration curve extending over a wide range of con-

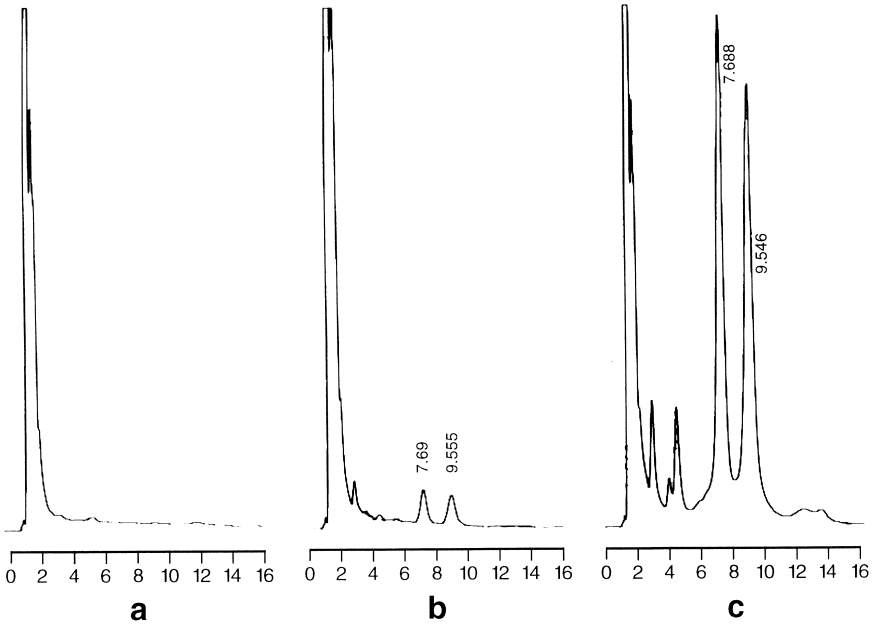

Fig. 3. Chromatograms of extracted plasma, collected from a rabbit 1 (c) and 6 (b) h after the last of 14 daily doses of 4 mg/kg of liposomal nystatin, compared with plasma from the same animal before administration of drug (a). The sum of the peak areas of the two major peaks of nystatin, which eluted at 7.5–8.5 and 9.5–10.5 min, was used for quantitation. The concentrations of nystatin in plasma were 12.78 (c) and 0.939 μg/ml (b), respectively.

centrations might have compromised accuracy and precision at the extremes of the range [15].

Within-day and between-day precision and accuracy of the method are presented in Tables 4 and 5, respectively. The within-day precision in rabbit plasma as measured by the coefficient of variation of the daily mean ($n=6$) was better than 9.5% for all four control concentrations except for one instance, where it was 13.1%. Between-day or overall precision showed a mean coefficient of variation of 4.4%, 4.0%, 9.5%, and 7.2% ($n=18$) for the 50, 5, 1.25, and 0.2 μg/ml nystatin controls, respectively.

The accuracy was determined by comparing the means of the observed concentrations with the nominal concentrations of nystatin in the plasma controls. Within-day ($n=6$) and between day ($n=18$) accuracies were within 6% of their expected values.

Incorporation of an internal standard for quantitation may have further optimized accuracy and precision. P-nitrophenol, tested initially for use as internal standard, was abandoned due to chromatographic interferences with the initial solvent complex and extreme light sensitivity. However, preliminary data from the manufacturer of liposomal nystatin suggest that natamycin, a tetraene with an absorbance maximum of 304–306 nm, may be a more useful candidate.

3.4. Lower limit of quantitation

The lower limit of quantitation (LOQ) was defined as the lowest quantity of analyte determined with a precision and an accuracy of ≤20%. Six replicates of the lowest standard concentration (0.050 μg/ml) were analyzed to evaluate the LOQ. At the LOQ, the percent variation of the measured concentrations was 14.36%, and the deviation of the mean of the measured concentrations from their nominal value was 10%.

3.5. Parallelism

A control plasma pool exceeding the upper limit of the standard curve containing 75 μg/ml nystatin

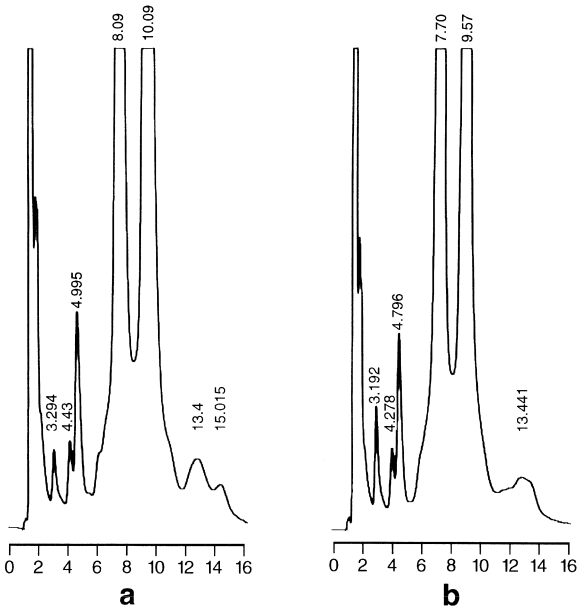

Fig. 4. Chromatogram of extracted normal rabbit plasma spiked to 50 μg/ml with bulk nystatin (a) and for comparison, that of an extracted plasma sample obtained from a rabbit immediately after infusion of 4 mg/kg of liposomal nystatin, containing 42.8 μg/ml of nystatin (b). At these comparatively high concentrations, at least seven different peaks could be identified in both plasma spiked with bulk nystatin and plasma obtained from rabbits dosed with liposomal nystatin.

was prepared. Six separate aliquots from this pool were extracted. The resulting methanolic solutions were submitted to assay undiluted and 1:1 (v/v) diluted in mobile phase. The mean ($n=6$) values for both preparations were within ±1% of their expected values. The precision was better than 2.7% coefficient of variation ($n=6$) at both preparations.

Table 1
Concomitant drugs which do not interfere with nystatin chromatography

Acetaminophen	Ethosuximide	Procainamide
Amikacin	Gentamicin	Propanolol
Amitryptiline	Lidocaine	Quinidine
Carbamazepine	Lithium	Salicylate
Ceftazidime	Methotrexate	Theophylline
Cyclosporine	Phenobarbital	Tobramycin
Digoxin	Phenytoin	Valproic Acid
Disopyramide	Primidone	Vancomycin

Table 2
Calibration curve parameters for nystatin in rabbit plasma

Day	Curve	Slope	Intercept	r^2
1	1a	2.16×10^5	-1.01×10^5	1.000
	2a	2.11×10^5	-1.06×10^5	0.999
2	3a	2.06×10^5	-0.34×10^5	1.000
	4a	2.19×10^5	-1.12×10^5	0.999
3	5a	2.10×10^5	-0.78×10^5	0.999
	6a	2.11×10^5	-0.87×10^5	0.999
1	1b	1.69×10^5	-3.97×10^3	0.998
	2b	1.62×10^5	-2.31×10^3	1.000
2	3b	1.64×10^5	-5.15×10^3	0.996
	4b	1.69×10^5	-4.61×10^3	0.998
3	5b	1.65×10^5	-4.37×10^3	0.992
	6b	1.57×10^5	-4.26×10^3	0.999

3.6. Absolute recoveries

The absolute recoveries of free nystatin and liposomal nystatin from rabbit plasma were determined in duplicate by dividing the slopes of the calibration standard curves in plasma after extraction by the slopes of the calibration standard of free nystatin in mobile phase directly injected onto the HPLC system. The absolute mean recovery of free nystatin and liposomal nystatin were 30.73% (range, 26.63–34.75) and 35.12% (range, 33.15–38.05), respectively. However, considering the dilution of plasma samples by the addition of methanol in a 2:1 (v/v) ratio, these recoveries correspond to an approximately complete extraction of compound from plasma samples.

3.7. Effects of centrifugation

Potential effects of centrifugation on the recovery of liposomal nystatin from whole blood were assessed at two concentrations. After determination of the hematocrit, triplicate aliquots of 3 ml of heparinized (10 IU heparin sodium USP per 2 ml of blood) whole blood obtained from a human volunteer were spiked with liposomal nystatin and equilibrated on a rotator for 1 h at 37°C. Thereafter, from each sample, 0.5 ml of whole blood was transferred into an 1.5 ml

Table 3
Calibration curve data for nystatin in plasma

Calibration curve	Spiked concentration (μg/ml)	Mean calculated concentration (μg/ml)	SD	C.V. (%)	Accuracy (%)
High range	50.000	50.235	0.358	0.71	+0.47
	25.000	24.600	0.795	3.23	−1.60
	12.500	12.588	0.164	1.31	+0.70
	6.250	5.706	0.373	6.54	−8.71
	3.125	3.286	0.181	5.51	+5.14
	1.562	1.617	0.111	6.86	+3.53
	0.000	0.000	0.000	0.00	0.00
Low range	1.562	1.586	0.018	1.13	+1.55
	0.781	0.744	0.041	5.57	−4.70
	0.390	0.377	0.021	5.47	−3.26
	0.195	0.192	0.011	5.72	−1.67
	0.097	0.099	0.006	5.86	+1.83
	0.048	0.057	0.007	11.64	+18.40
	0.000	0.000	0.000	0.00	0.00

Table 4
Within-day variations of the assay for nystatin in rabbit plasma

Calibration curve	Spiked concentration (μg/ml)	Mean calculated concentration (μg/ml)	*n*	SD	C.V. (%)	Accuracy (%)
High range	50.000	49.599	6	1.560	3.146	−0.9
		47.785	6	2.612	5.467	−4.5
		48.626	6	2.026	4.166	−2.8
	5.000	4.738	6	0.206	4.350	−6.3
		4.982	6	0.143	2.868	−0.4
		5.021	6	0.110	2.200	+0.4
Low range	1.250	1.321	6	0.116	8.780	+5.6
		1.296	6	0.170	13.093	+3.6
		1.324	6	0.102	7.669	+5.9
	0.200	0.200	5	0.013	6.500	±0.0
		0.207	6	0.008	3.890	+3.5
		0.192	6	0.018	9.464	−4.0

Table 5
Between-day variations of the assay for nystatin in rabbit plasma[a]

Calibration curve	Spiked concentration (μg/ml)	Mean calculated concentration (μg/ml)	*n*	SD	C.V. (%)	Accuracy (%)
High range	50.000	48.687	18	2.147	4.410	−2.7
	5.000	4.914	18	0.197	4.005	−1.8
Low range	1.250	1.314	18	0.125	9.517	+5.1
	0.200	0.200	17	0.015	7.267	±0.0

[a] Results of validation studies over a three-day period with six determinations per day

polypropylene tube and extracted with 1:2 (v/v) of methanol. A further 1.5 ml were transferred into a second polypropylene tube and centrifuged at 3000 *g* for 15 min. Then, 0.5 ml of the resulting plasma supernatant was submitted to extraction with 1:2 (v/v) of methanol. The cell pellet was weighed after removal of remaining plasma, and resuspended and extracted with 1:2 (w/w) of methanol.

Relative recoveries were calculated using the mean area response of extracted whole blood, serum, and pellet versus that of spiked and extracted human volunteer plasma as reference standard. The calculated value for 100% recovery of drug in plasma derived after centrifugation of spiked whole blood was calculated by using the following equation:

Calculated 100% recovery [area] = 1.00/(1.00 − hkt) × reference standard [area]

In comparison to plasma spiked to identical concentrations and submitted to the same extraction method, the mean relative recovery of liposomal nystatin from whole heparinized blood was 69.88 and 78.33% at the 20 and 2 μg/ml concentration, respectively (Table 6). In contrast, after centrifugation and extraction from plasma, recoveries were considerably higher with 87.33 and 96.57%, respectively, of the calculated value for complete recovery. These results confirm that only minor amounts of compound sediment with the cellular fraction of whole blood during standard centrifugation procedures utilized for obtaining plasma. The lower recovery from whole blood observed in these experiments could be due to methanol-induced clumping of blood cells and entrapment of drug in the cellular fraction of the specimens.

3.8. Process stability

Process stability was assessed by extracting 4 sets of plasma control samples (0.6, 1.2, 5.0, and 20 mg/ml; 3 samples per concentration). Aliquots of the extracted samples were run immediately after preparation and again after 3 h of storage at room temperature. The mean values for accuracies after storage at room temperature were within 8.2% of their expected values (−7.0; −8.2; −3.70; and −1.30%; coefficient of variation: 2.76; 2.75; 5.73; and 2.48%, respectively).

Stability of extracted samples maintained at 4°C in the autosampler was tested by extracting 4 sets of plasma control samples (0.6, 1.2, 2.0, and 20 mg/ml; 3 samples per concentration). Aliquots of the extracted samples were run immediately after preparation and again after 12 and 24 h of storage at 4°C in the autosampler. Maintaining extracted samples at

Table 6
Effects of centrifugation on the recovery of liposomal nystatin from blood samples[a]

Reference[b] (peak area±SD)	Recovery from whole blood, plasma supernatant, and buffy coat (peak area±SD)			
	Whole blood	Plasma	Pellet	Plasma + pellet
Spike: 20 μg/ml	Spike: 20 μg/ml			
2724882 ±105443 [100%]	1904276±86886 [69.88%][c]	4616563±119354 [87.33%][d]	314640±23803 [6.38%][e]	4931203±121660 [93.28%][f]
Spike: 2 μg/ml	Spike: 2 μg/ml			
223463 ±8625 [100%]	175055±15136 [78.33%][c]	418689±22474 [96.57%][d]	21670±3023 [4.92%][e]	440353±22128 [101.56%][f]

[a] All values represent the mean ±SD of three separately spiked samples.
[b] Normal human plasma, spiked to the indicated concentration and extracted 1:2 in MeOH.
[c] % recovery as compared to the reference value in plasma.
[d] % recovery as compared to the projected value for 100% recovery in plasma after centrifugation.
[e] % recovery in the cell pellet as compared to the total recovery (plasma plus pellet) after centrifugation.
[f] % total recovery as compared to the projected value for 100% recovery in plasma after centrifugation.

Table 7
Stability of extracted nystatin maintained at 4°C in the autosampler

Calibration curve	Spiked concentration (μg/ml)	Concentration found (μg/ml)[a]		
		Baseline	12 h	24 h
High range	20.000	18.196±0.832	18.624±0.899	19.469±0.826
	2.000	2.037±0.014	2.055±0.024	2.037±0.108
Low range	1.200	1.257±0.088	1.268±0.078	1.298±0.194
	0.600	0.598±0.031	0.598±0.007	0.598±0.025

[a] All values represent the mean±SD of three samples each.

4°C in the autosampler for up to 24 h had little effect on the accuracy of the results (Table 7). The coefficient of variation was within 7.00% except for 1.2 μg/ml at 24 h, where it was 14.94%.

3.9. Storage stability

The stability of nystatin was tested with spiked plasma samples (0.6, 1.2, 5.0, and 20 mg/ml, respectively) stored in polypropylene tubes at −80°C. They were assayed in triplicate on the day of preparation (baseline) and thereafter at 1 month and 3 months of storage. Nystatin was stable in plasma for at least three months when stored at −80°C (Table 8). Mean accuracies were within 9% of the expected values, and % variation was equal to or less than 6.00 and 12.30 respectively, for 1 and 3 months of storage.

Stability at −80°C was similarly tested by re-analysis of 20 randomly selected plasma samples from rabbits dosed with liposomal nystatin ten months after the initial analysis. Samples were grouped into three concentration ranges: 60 to 10 μg/ml ($n=6$), 9 to 5 μg/ml ($n=6$), and 0.2 to 1.5 μg/ml ($n=8$). Mean accuracies at re-analysis for the three concentration ranges were 104.14% (SD, 7.48;% variation, 7.17), 100.40 (SD, 5.063;% variation, 5.043), and 103.99 (SD, 9.38;% variation, 9.020), respectively.

Stability was also tested by subjecting plasma control samples (spiked to 0.6, 1.2, 5.0, and 20 mg/ml, respectively) to three freeze/thaw cycles. Thawing and refreezing had little effect on accuracy and precision of the results: The mean ($n=3$) observed values were within 6.17% of the expected values, and % variation was 12.59, 5.98, 6.40, and 2.42 for the four assayed concentrations, respectively.

3.10. Column life span

At a column temperature of 30°C and a flow-rate of 2.00 ml/min, normal operating backpressure was approximately 135 bar. Over 500 injections from extracted plasma could be made before a significant increase in backpressure (≥160 bar) and subsequent loss of peak resolution was noted. Guard columns were replaced after approximately 200 injections.

Table 8
Stability of nystatin in rabbit plasma stored at −80°C

Calibration curve	Spiked concentration (μg/ml)	Concentration found (μg/ml)[a]		
		Baseline	1 month	3 months
High range	20.000	19.667±0.176	19.187±1.022	21.593±0.199
	5.000	4.897±0.239	4.822±0.082	4.853±0.333
Low range	1.200	1.217±0.042	1.219±0.047	1.253±0.114
	0.600	0.637±0.061	0.566±0.034	0.607±0.075

[a] All values represent the mean±SD of three samples each.

3.11. Preclinical studies

Thus far, the method has been applied to two completed [10,16] and two currently ongoing preclinical pharmacokinetic and pharmacokinetic/pharmacodynamic studies. Plasma samples were extracted as described. After collection and separation of the plasma, samples were stored at −80°C until their analysis. A representative plasma concentration vs. time profile from a rabbit dosed with liposomal nystatin is shown in Fig. 5. As previously observed with multilamellar liposomal drug formulations [17,18], after intravenous administration of 4 mg/kg over 10 min, liposomal nystatin achieved relatively high peak plasma levels and was then rapidly distributed and eliminated from plasma with a relatively short half-life. Of note, plasma pharmacokinetic parameters in the rabbits [16] were very similar to those measured in the blood of patients [19], which provides a valid basis for understanding the drug's pharmacokinetic/pharmacodynamic relationships in animal models and in humans. Based on the observation that concentrations in plasma were approximately twice as high as corresponding levels in blood (Table 6), and using the mean area under the concentration vs. time curve from zero to 24 h (AUC_{0-24}) for comparison, a 4 mg/kg dose in the rabbit would approximately correspond to a 2 mg/kg dose in humans (the target dose in ongoing clinical trials), and a 6 mg/kg dose to a 3 mg/kg dose, respectively (AUC_{0-24}, 30 vs. 15 μg h/ml and 71 vs. 27 μg h/ml, respectively). Similarly, for the indicated dosages, other key pharmacokinetic parameters appear to be of the same magnitude: Mean volume of distribution, 0.18–0.22 l/kg in rabbits vs. 0.51–0.84 l/kg in patients; clearance, 0.096–0.135 vs. 0.120–0.192 l/(h kg); and half-life, 1.45–1.23 h vs. 1.9–3.6 h, respectively.

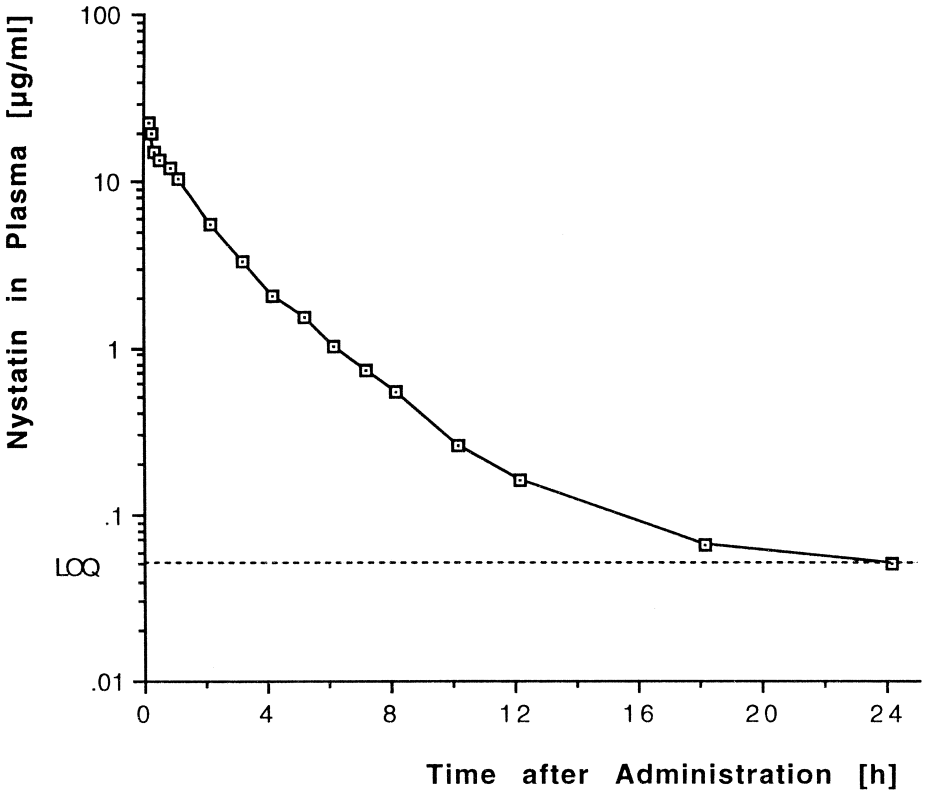

Fig. 5. Plasma-concentration vs. time profile for nystatin in a rabbit from an experimental pharmacokinetic study following the last of 14 daily intravenous administrations of 4 mg/kg of liposomal nystatin over 10 min.

The method was also used for determination of drug concentrations in tissues and body fluids. While the extraction procedure for body fluids was identical to that utilized for plasma, direct 1:2 (w/w) homogenization in methanol was used for extraction of nystatin from solid tissues. Seven-point standard curves in methanolic homogenates from brain, lung, liver, spleen and kidney homogenates were linear with r^2-values ≥ 0.997. Accuracies of triplicate quality controls at both ends of the calibration standards were within 10% (mean, 4.71; range, 2–10) of their expected values, and precision was better than 11% coefficient of variation (mean, 4.17; range, 0.91–10.71). Similarly, seven-point standard curves from cerebrospinal fluid and urine had r^2-values of 0.999 and 0.984, respectively, and precision and accuracies were within 14%.

4. Conclusions

The assay procedure presented in this report provides a simple and rapid means for the accurate and precise quantitation of liposomal nystatin in rabbit plasma. The robustness of the assay has been demonstrated in assaying several hundred specimens from rabbits treated with liposomal nystatin, and, with minor modifications, in human subjects. The method can also be used for the analysis of nystatin in tissues and human plasma. Potential modifications of the method may include simple precipitation/centrifugation for sample preparation, the use of an internal standard, and weighed linear regression for generation of a single calibration curve with a tighter range of concentrations.

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