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Short communication

High-performance liquid chromatographic determination of amphotericin B in plasma and tissue Application to pharmacokinetic and tissue distribution studies in rats

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Abstract

A sensitive HPLC method with piroxicam as internal standard was developed for assaying amphotericin B in plasma and tissue. An Ultrabase-C_{18} column and a simple mobile phase consisting of an acetonitrile–acetic acid (10%)–water (41:43:16) mixture were used. The flow-rate was 1 ml/min and the effluent was monitored at 405 nm. The linearity of the assay method was up to 1000 ng/ml and 100 μg/g for plasma and tissue, respectively. Intra-day and inter-day RSDs were below 10% for all the sample types. This HPLC assay has been applied to determine amphotericin B in plasma and tissue samples taken during pharmacokinetic and tissue distribution studies in rats.

Keywords: Pharmaceutical analysis; Amphotericins; Antibiotics; Piroxicam

1. Introduction

Amphotericin B, an antifungal agent with a polyene structure, may be considered the antibiotic of choice for treatment of invasive and disseminated fungal infections [1]. However, its clinical use is limited by the considerable treatment-associated toxicity of the drug, the most serious being nephrotoxicity [2–5]. Thereby, new formulations of amphotericin B are being studied with the aim of improving the therapeutic index of the drug by altering its plasma and tissue distribution profiles [6–8]. In order to characterize the pharmacokinetics and tissue distribution of amphotericin B when it is administered in new dosage forms, the drug levels in the plasma as well as the tissue need to be measured and therefore, a sensitive, accurate and reproducible analytical method is necessary.

Numerous chromatographic methods have been previously published for the analysis of amphotericin B in biological matrices (plasma, urine, tissue, etc.). Some of these methods describe procedures for analyzing amphotericin B after deproteinization with methanol or acetonitrile, and direct injection of the supernatant onto the chromatographic column [9–14]. However, most of them present insufficiently sensitivity, inadequate reproducibility, low recovery or chromatographic interferences by endogenous compounds, which limit their application in pharmacokinetic and tissue distribution studies of amphotericin B. Other methods show adequate sensitivity and precision but involve solid-phase extraction [15–17] which notably increases the time of analysis.

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In this paper, a rapid, simple, accurate, reproducible and sensitive method is described for the assay of amphotericin B in plasma and tissue by HPLC–UV, using piroxicam as internal standard. The assay involves deproteinization and the application of an Ultrabase C_{18} column for the chromatographic separation.

The use of an Ultrabase C_{18} column greatly improves the resolution of the amphotericin B peak in comparison with other columns [9–12]. On the other hand, this method uses piroxicam as internal standard [15] which is readily available compared with 3-nitrophenol [12] and N-acetylamphotericin B [18]. Moreover, its absorbency wavelength is similar to that of amphotericin B and so both compounds may be detected at 405 nm, in contrast with the method developed by Polikandritou Lambros et al. [19] which uses two detectors in series set at two different wavelengths (383 and 303 nm) to detect amphotericin B and natamycin (internal standard). Although piroxicam is chemically very unlike to amphotericin B (Fig. 1), its retention time and solubility in the mobile phase makes it appropriate as the internal standard.

This method offers other advantages such as the simplicity of the sample preparation procedure which compared with solid-phase extraction gives the assay rapidity and moreover, the detection and quantitation limits obtained are lower than those of the other published methods [10,12].

The developed HPLC assay method has been successfully applied for determination of amphotericin B in plasma and tissue samples taken during pharmacokinetic and tissue distribution studies in rats.

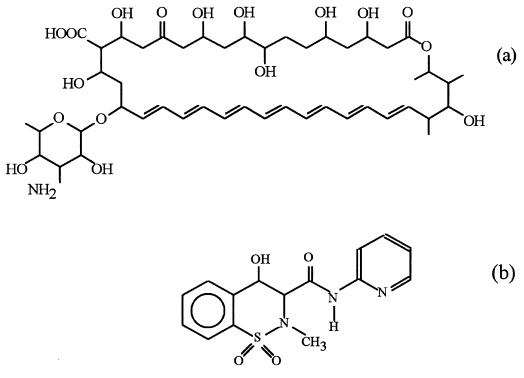

Fig. 1. Chemical structures of amphotericin B (a) and piroxicam (b).

2. Experimental

2.1. Chemicals, reagents and solutions

Amphotericin B (AmB) was a gift from Nextar (Madrid, Spain) and piroxicam (internal standard) was supplied by Boral Química (Barcelona, Spain). Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid (HPLC grade) and dimethyl sulfoxide (DMSO, reagent grade) were obtained from Merck (Darmstadt, Germany).

Stock solutions of AmB (1 mg/ml) and piroxicam (1 mg/ml) were prepared in a mixture of DMSO–methanol (1:1, v/v) and acetonitrile, respectively. Stock solution of AmB was stored at −20°C; under these conditions the drug was found to be stable for at least one month. Working solutions of this antibiotic were obtained by dilution from stock solution with methanol for use as calibration standards by addition to plasma and tissue homogenates.

2.2. Chromatography

The HPLC equipment used consisted of a Waters 600E pump, a Waters 700 Satellite WISP autosampler and a Waters 486 ultraviolet detector set at 405 nm. The system was equipped with a Waters integrator (Waters Assoc., Milford, MA, USA).

Separation was achieved using an Ultrabase C_{18} reversed-phase column (250 mm×4.6 mm I.D., 5 μm particle size) (Scharlau, Barcelona, Spain), preceded by a guard column (45 mm×4.6 mm I.D.) filled with pellicular C_{18} (5 μm particle size) (Perkin-Elmer, Norwalk, CT, USA). Mobile phase was acetonitrile–acetic acid (10%)–water (41:43:16) at a flow-rate of 1 ml/min. The chromatography was carried out at room temperature.

2.3. Sample preparation procedures

2.3.1. Plasma

To a 100 μl plasma sample, an adequate internal standard amount and 200 μl of acetonitrile were added. After vortex mixing (30 s), the sample was

centrifuged at 10 000 rpm for 10 min. The supernatant was transferred to a glass conical tube and evaporated to dryness in a vortex evaporator (60°C) (Buchler Instruments, Fairfield, NJ, USA). The residue was finally dissolved in 250 μl of the chromatographic eluent and an aliquot of this solution (100 μl) was injected into the HPLC system after Millipore Millex-LS filtration.

2.3.2. Tissue

To a 0.5 g tissue sample (liver, spleen or kidney), 2 ml of doubly distilled water and an adequate amount of internal standard were added. After that, the sample was homogenized by using an ultraturrax-T25 (24 000 rpm, 3 min) (Janke & Kunkel, Staufen, Germany). An aliquot of the homogenate (300 μl) was transferred to a glass conical tube and 900 μl of methanol were added. The mixture was vortexed (30 s) and centrifuged at 10 000 rpm for 10 min. The supernatant was filtered and an aliquot (100 μl) was injected into the chromatograph.

2.4. Recovery

The recoveries of AmB from plasma, liver, spleen and kidney were determined by spiking an equal amount of the drug into the corresponding blank sample and the mobile phase for plasma and methanol for tissue. Recoveries at three concentration levels were studied in quintuplicate for each sample type.

Percentage recovery was calculated by comparing the absolute responses (peak area) of AmB from sample extracts to the absolute responses (peak area) of non-extracted standards (AmB in mobile phase and methanol for plasma and tissue, respectively).

2.5. Linearity, precision and accuracy

Calibration curves of AmB in plasma and tissue were established over the following concentration ranges: 10–1000 ng/ml for plasma, 2.5–100 μg/g for liver and 5–100 μg/g for spleen and kidney. At least seven data points were utilized to construct the curves. Peak area ratios of AmB and internal standard versus the corresponding drug concentrations were plotted.

The detection and quantitation limits were estimated by repeated analysis of the blank sample of plasma and tissue homogenates (liver, spleen and kidney).

Precision (intra-day and inter-day variation) was evaluated by analyzing five replicate plasma and tissue samples at the following concentrations: 25, 100 and 1000 ng/ml for plasma, 5, 25 and 100 μg/g for liver, and 7.5, 50 and 100 μg/g for spleen and kidney. The variability was expressed as the relative standard deviation (RSD). To be acceptable, the values should be less than 15% at all concentrations [20].

Accuracy of the assay method was calculated from the same samples as those used for intra-day variation studies. To be acceptable, the values should be within 15% at all concentrations [20].

2.6. Application of the method to pharmacokinetic and tissue distribution studies in rats

As an example, the proposed HPLC method was applied to determine the plasma and tissue concentration-time profiles of AmB following intravenous bolus administration of Fungizone (1 mg/kg) in rats (33 Wistar males, 225–250 g). Blood and tissue samples were obtained at 5, 10, 15, 30 min and 1, 3, 6, 10, 24, 48 and 72 h. The plasma was separated by centrifugation (10 000 rpm 10 min) and the liver, spleen and kidneys were removed, dried and weigh. All biological specimens were stored at −20°C until the time of analysis; under these conditions AmB was found to be stable in these biological matrices.

The plasma concentration-time profile of AmB was described using a two-exponential equation: $C = A_o e^{-\alpha t} + B_o e^{-\beta t}$. The equation was fitted to the data using the non-linear least squares regression program WinNonlin (WinNonlin, Scientific Consulting, Apex, NC, USA).

Basic pharmacokinetic parameters such as total plasma clearance (Cl), volume of distribution at steady state (V_{ss}), area under the curve (AUC) and half-life of the first and terminal phases ($t_{1/2\alpha}$ and $t_{1/2\beta}$, respectively) were calculated according to standard procedures using the coefficients and the exponents of the fitted function.

3. Results and discussion

The Ultrabase C_{18} column and the simple mobile phase used were found to be appropriate for the analysis of AmB. Under the assay conditions described, retention times for AmB and the internal standard were around 5.8 and 7.6 min, respectively. In Fig. 2 the chromatograms obtained for drug-free rat samples (plasma and liver) and post-dose rat samples obtained after intravenous administration of the drug are shown. No peaks with retention times similar to those of AmB and the internal standard were present in blank rat samples. Chromatograms of spleen and kidney specimens were similar to those of liver samples.

Studies using the Nucleosil-120 C_{18} (125 mm×4 mm I.D., 5 μm particle size) (Scharlau) and Kromasil-100 C_{18} (125 mm×4 mm I.D., 5 μm particle size) (Teknokroma) columns, resulted in poor band symmetry and provided undesirably broad peaks for AmB. However, good peak symmetry and minimal band spreading were obtained with the Ultrabase C_{18} column.

Different ratios and flow-rates of the mobile phase were studied in order to shorten the retention times of AmB and the internal standard, however the optimal separation was obtained when the ratio was 41:43:16 for acetonitrile–acetic acid (10%)–water, and the flow-rate 1 ml/min.

Recovery of AmB averaged 84% from plasma at concentrations of 25–1000 ng/ml, 92% from liver at concentrations of 5–100 μg/g of tissue, and 87% and 96% from spleen and kidney, respectively, at concentrations of 7.5–100 μg/g of tissue. The results are summarized in Table 1.

The peak area ratios AmB/internal standard showed a linear relationship to drug concentrations within the range 10 to 1000 ng/ml for plasma, 2.5 to 100 μg/g for liver and 5 to 100 μg/g for spleen and kidney. Quantitation based on peak-area ratios between amphotericin B and internal standard was found to yield more consistent and reproducible results than peak-height ratio calculations. The parameters of the calibration curves were: $y = 0.0014x - 0.0072$ for plasma, $y = 0.015x + 0.018$ for liver, $y = 0.011x - 0.023$ for spleen and $y = 0.013x - 0.021$ for kidney (x = concentration of AmB; y = peak area ratio AmB/internal standard). The results of linear regression analysis show that the correlation coefficients of the calibration curves for all sample types were ~0.999.

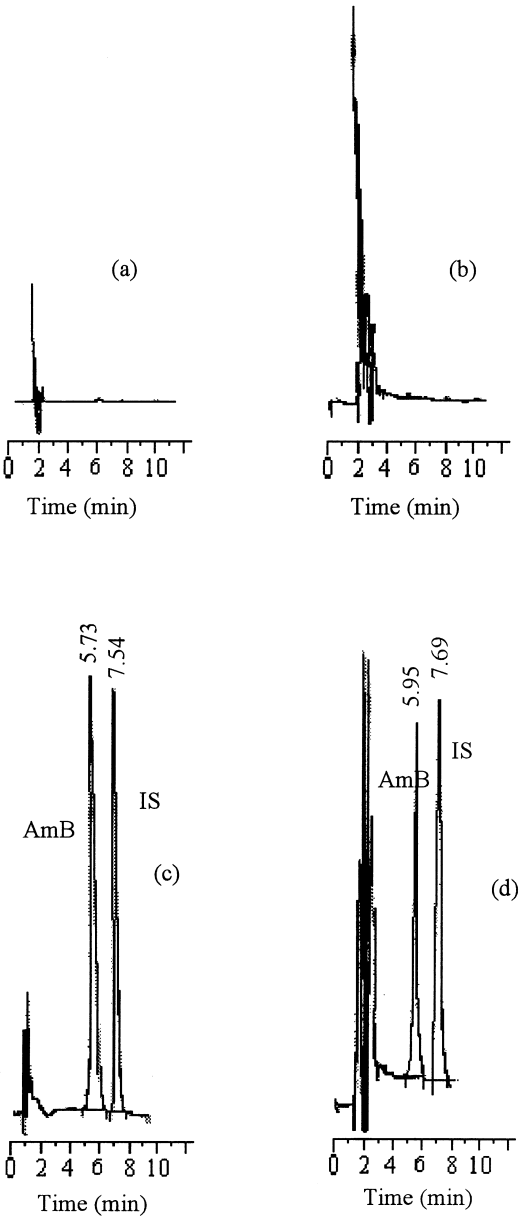

Fig. 2. Typical chromatograms of drug-free plasma (a) and liver (b) samples. Chromatograms of post-dose plasma (c) and liver (d) specimens obtained 1 h after intravenous bolus administration of Fungizone (1 mg/kg) in rat. Amb = Amphotericin B; IS = internal standard.

Table 1
Recoveries of amphotericin B from plasma and tissue

	Recovery (%): mean±SD ($n=5$)			
	Plasma	Liver	Spleen	Kidney
High	78.4±1.3	82.1±5.2	83.2±5.3	93.4±3.3
Medium	97.0±9.4	96.3±0.8	88.7±0.9	96.6±2.0
Low	76.3±9.9	97.2±3.7	85.3±7.5	98.8±9.3

The detection limits of the assay were found to be 2.0 ng/ml for plasma, 0.62 μg/g for liver, 0.42 μg/g for spleen and 1.96 μg/g for kidney. The estimated limits of quantitation were 6.6 ng/ml for plasma, 2.04 μg/g for liver, 1.5 μg/g for spleen and 6.5 μg/g for kidney. These limits of detection and quantitation were validated obtaining coefficients of variation <15%.

The intra-day variabilities of the assay method for plasma, liver, spleen and kidney samples are shown in Table 2 and the corresponding inter-day variabilities in Table 3. The data indicate that the assay method is reproducible within the same day and within different days; RSDs are less than 10% for the all sample types over the concentration ranges assayed.

The accuracy of the assay method in determining AmB concentrations in spiked plasma and tissue specimens is presented in Table 4. The deviation from theoretical values is below 11% at all concentration levels studied for each sample type.

Following intravenous bolus administration of Fungizone in rats, the plasma concentration-time curve of the drug was adequately described by a biexponential equation: $C=0.2e^{-3.6t}+0.13e^{-0.03t}$. In Fig. 3 the plasma and tissue concentration versus time curves of AmB in rats following a single

Table 2
Intra-day variabilities of the HPLC assay for amphotericin B

	Concentration found (mean±SD; $n=5$) and RSD							
	Plasma (ng/ml)	RSD (%)	Liver (μg/g)	RSD (%)	Spleen (μg/g)	RSD (%)	Kidney (μg/g)	RSD (%)
High	1045.9±25.5	2.4	96.5±1.9	1.9	110.8±6.9	6.2	101.4±3.5	3.5
Medium	97.4±2.5	2.5	24.9±0.3	1.2	48.0±2.5	5.1	45.4±1.6	3.5
Low	26.6±1.6	6.1	5.1±0.5	9.4	7.7±0.7	9.3	7.7±0.3	4.7

Table 3
Inter-day variabilities of the HPLC assay for amphotericin B

	Concentration found (mean±SD; $n=5$) and RSD							
	Plasma (ng/ml)	RSD (%)	Liver (μg/g)	RSD (%)	Spleen (μg/g)	RSD (%)	Kidney (μg/g)	RSD (%)
High	899.1±74.7	8.3	102.4±4.0	3.9	106.2±2.7	2.5	100.7±1.4	1.4
Medium	92.3±9.2	9.9	24.1±1.7	7.0	50.9±1.6	3.2	48.5±1.7	3.6
Low	24.5±0.6	2.4	5.3±0.5	9.3	7.9±0.8	9.8	7.9±0.5	6.5

Table 4
Accuracy of the HPLC assay for amphotericin B

	Concentration found (mean±SD; $n=5$) and RSD							
	Plasma (ng/ml)	Deviation (%)	Liver (μg/g)	Deviation (%)	Spleen (μg/g)	Deviation (%)	Kidney (μg/g)	Deviation (%)
High	1045.9±25.5	4.5	96.5±1.9	−3.5	110.8±6.9	10.8	101.4±3.5	1.4
Medium	97.4±2.5	−2.6	24.9±0.3	−0.4	48.0±2.5	−4.0	45.4±1.6	−9.2
Low	26.6±1.6	6.4	5.1±0.5	2.0	7.7±0.7	2.7	7.7±0.3	2.7

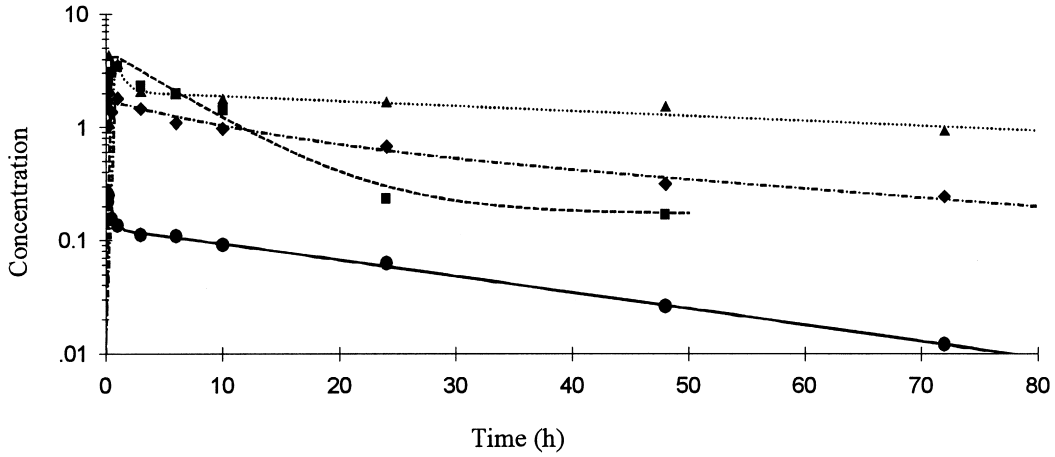

Fig. 3. Amphotericin-B plasma (●), liver (■), kidney (▲) and spleen (♦) concentration-time profiles following intravenous bolus administration of Fungizone to rats (1 mg/kg). The lines represent the computer fit to all individual data. The dots denote the mean concentrations ($n=3$).

intravenous administration of 1 mg/kg Fungizone are shown. The pharmacokinetic parameter estimates of the drug were: $Cl=0.015\pm0.005$ l h^{-1} kg^{-1}, $V_{ss}=0.18\pm0.03$ l kg^{-1}, $AUC=1.01\pm0.04$ ng hl^{-1} kg^{-1}, $t_{1/2\alpha}=0.18\pm0.07$ h and $t_{1/2\beta}=20.5\pm0.9$ h.

The method described is sensitive enough for the quantitative determination of AmB in plasma and tissue. It has been successfully applied to pharmacokinetic and distribution studies of this drug in rats. Moreover, the rapidity, simplicity and efficacy of the developed method as well as the relatively short retention times of AmB and the internal standard permit the analysis of a large number of samples in a short time providing a fast and inexpensive method for therapeutic monitoring in clinical laboratories.

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