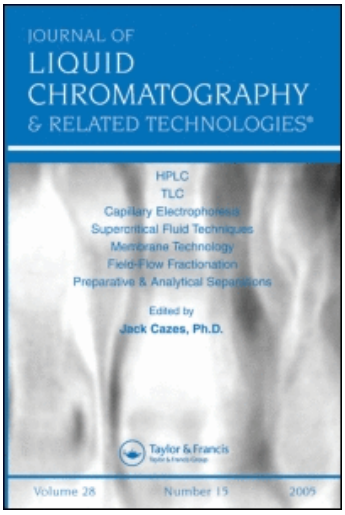

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IMPROVED ASSAY FOR SERUM AMPHOTERICIN-B BY FAST HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A selective and sensitive method for the determination of amphotericin B in dog serum, after deproteinization, using high performance liquid chromatography HPLC with UV detection at 382 nm was developed. After protein precipitation with methanol, 5 µl of the supernatant was injected onto a reversed phase C_{18} column (1.5µm, 33 x 4.6 mm ID), with a mobile phase composed of 0.05 M sodium acetate - acetonitrile - methanol (40:30:30, v/v). The analytical recovery of amphotericin B in serum was 92.30%. The linearity of the assay method was verified up to 15 µg/mL of amphotericin B. The assay was reproducible with satisfactory intra-day and inter-day coefficients of variations (CV < 3%). The sensitivity of the method was ≤0.625 ng of amphotericin B for serum. The method was successfully employed for the pharmacokinetic analysis of amphotericin B in dogs following intravenous administration of liposomal amphotericin B (AmBisome).

INTRODUCTION

Amphotericin B, a macrocyclic polyene antibiotic, has a broad spectrum of action against fungal (*Aspergillus, Candida, Cryptococcus, Blastomyces, Histoplasma*) and protozoan (*Leishmania*) pathogens.[1] This drug binds to ergosterol, the principal sterol in the membrane of susceptible cells, causing impairment of membrane barrier function. Administration of conventional colloidal dispersion in sodium deoxycholate (Fungizone) is associated with severe side effects in man and animals. Liposomal preparations of Amphotericin B (AmBisome) are much better tolerated and much higher doses can be administered without toxic reactions.[2]

High efficacy of liposomal amphotericin B against *Leishmania infantum*, the agent of human and canine visceral leishmanians, was shown in experimental[3] and clinical[4] studies. Recently, the role of this drug for the treatment of the disease in dogs is being investigated by dose-searching clinical trials.[5] In this animal, however, the pharmacokinetic behaviour of liposomal amphotericin B needs to be elucidated.

Several high performance liquid chromatographic (HPLC) assays have been reported recently, which, offer faster and more accurate and reproducible alternatives to bioassays for both pharmacokinetic studies and routine clinical use of amphotericin B.[6-16] HPLC offers improved sensitivity and specificity and is easier to standardise than bioassays are.[1] The chromatographic methods reported recently in the literature use conventional and short columns (30 - 60 mm length),[11,13] several of these also demonstrate a good sensitivity,[14] but use large volume of sample (50 - 150 µL). Repeated sampling of blood from small animals requires small amounts of plasma to avoid volume depletion. A convenient, sensitive method for measurement of AmB in small amounts of serum was needed.

Recently, little columns packed with non porous silica microspheres with diameters of 1.5 µm became available. These columns achieve better resolution and faster separation than that of conventional columns and only a small volume of sample is needed.

In this study, we have developed a sensitive, specific, accurate, and reproducible analytical method for the determination of AmB concentrations in serum samples. Using isocratic reversed phase method with 33 mm column (1.5 µm particle size) and adjustment of the mobile phase composition, it has been possible to separate and determine AmB in serum using a small volume of sample. This method has been successfully employed in pharmacokinetic studies using dog serum treated with liposomal AmB (AmBisome).

EXPERIMENTAL

Chemical and Reagents

Amphotericin B, reference standard was a gift from Bristol-Myers Squibb. (Sermoneta LT, Italy), sodium acetate, analytical reagent, was obtained from Carlo Erba (Milan, Italy), methanol and acetonitrile, HPLC-grade were obtained from Eurobase (Milan, Italy).

Chromatographic System

The HPLC system used for the analysis of all the samples and standards consisted of a Waters 600E HPLC pump (Waters, Milford, MA, USA), connected to autosampler system Waters 717 plus . Detection of amphotericin B was accomplished by Waters 994 photodiode array detector at a wavelength of 382 nm. Chromatograms, peak area, peak spectra, and peak purity parameters were obtained via Waters Millenium 2010 software.

Isocratic method of separation was achieved using MICRA NPS HPLC column, RP-C18 (1.5 μm, 33 x 4,6 mm ID), Micra Scientific (Northbrook, IL, USA). The mobile phase was 0.05 M sodium acetate - acetonitrile - methanol (40:30:30, v/v). The run time was 5 min with flow - rate of 0.7 mL/min.

Standard Solutions

Stock solutions of amphotericin B were prepared by dissolving amphotericin B authentic standard in DMSO (0.1 mg/mL). Serial dilutions of the stock solution were freshly prepared into appropriate concentrations using methanol. Standard curve samples of the sera were freshly prepared by spiking aliquots of the standard solutions of amphotericin B into the blank samples (200 μL of each serum sample). The concentration range of the final standard samples was 0.125 - 15.000 μg/mL of the supernatant.

Animal Treatment

Liposomal amphotericin B (AmBisome) was reconstituted in 5% dextrose and infused over 60 minutes via a peripheral vein of 4 dogs at the dose of 5 mg/kg bw. Blood was collected from a brachial vein at different time intervals

from 5 min. to 72 h after infusion. The blood was allowed to clot and the serum was separated by centrifugation at 1500 r.p.m. The samples were stored at -20°C pending assay.

Sample Preparation

Samples were deproteinized by adding 800μL of cold methanol to 200 μL of serum, were mixed by vortex-mixing and centrifuged at 5°C, 7000 r.p.m. for five minutes. The supernatant was filtrated through 0,45 μm Millipore filters. The 5 μL aliquots were injected into the column. The standards were assayed in the same manner.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, AmB was eluted approximately 2,5-3,0 min. Typical chromatograms of dog serum samples spiked with AmB and dog samples obtained after intravenous administration of AmBisome, are presented in Fig. 1. By minor adjustment of the composition of the mobile phase, it has been possible to determine amphotericin B with short retention time, which, permitted the analysis of a large number of samples obtained from pharmacokinetic studies.

The specificity of the method was assayed by comparing chromatograms of blank serum from dog. All chromatograms were free of interfering peaks. The Millenium PDA software determined the spectral homogeneity of the peak by comparing the peak apex spectrum against a spectrum of standard. Spectral analysis performed on the proposed AmB peak of methanolic standards, spiked sera and dog serum samples, obtained after AmBisome infusion, were essentially identical and exhibited spectral peaks normally associated with heptaenes (405, 382, 363, 344nm). This demonstrated that there was no interference from metabolites or endogenous substances. It also excluded the presence of the tetraene impurities of amphotericin B.

Recovery, Reproducibility, and Detection Limit

AmB was added to drug - free serum to provide concentrations of 0.25, 2.50 and 15.00 μg/mL. Recovery was determinated by replicate analysis (n=6) of the each of these serum pools. The peak areas obtained for AmB were compared with peak areas obtained by direct injection of working standard solution.

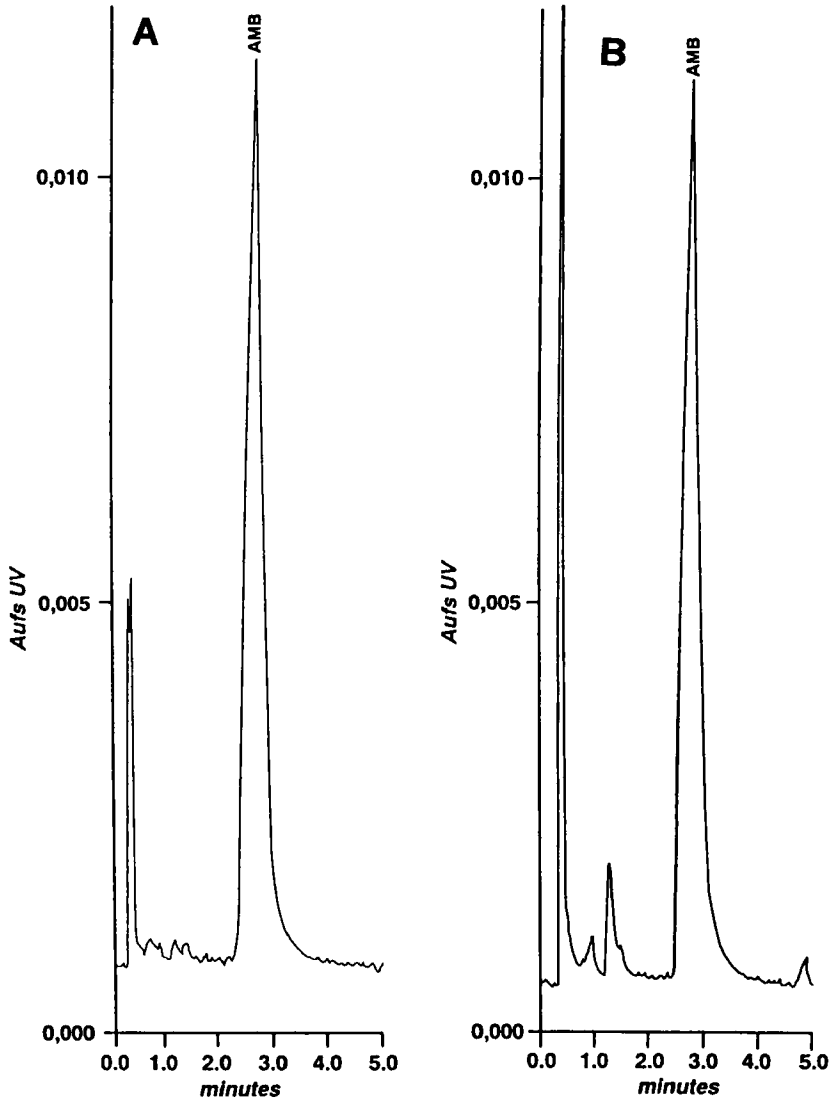

Figure 1. Typical chromatogram of amphotericin B in dog spiked serum at the final concentration of 10 μg/mL (A), and dog serum sample obtained after 60 min post infusion of AmBisome 5 mg/kg (B)

Analytical recoveries of added AmB from serum by the procedure of deproteinization (mean ± SD, n=6) were found to be 92.84% ± 2.37, 92.97% ± 1.79 and 91.11% ± 2.54.

The method was validated by performing replicate analysis (n=5) of pooled dog serum spiked with AmB in concentrations all over the studied range, on four separate days. Concentrations were determined using the respective standard curves prepared and checked on the day of the analysis. The results of the linear regression analysis showed that assay method had a verified linearity from 0.125 to 15.000 μg/mL of AMB with correlation coefficients of 0.9999 and errors of estimation ≤ 0.035. These levels were sufficiently high for the determination of AmB in serum of dog following intravenous administration of liposomal AmB. Y - intercept (0.029 ± 0.004) of calibration curves were calculated by standard statistical methods. Linear regression analysis was used to define accuracy by comparing the experimental values obtained for the AmB reference samples (y) with their theoretical values (x).

The precision and the overall accuracy of the method are presented in Table 1. The accuracy of the assay was defined as the mean of the absolute values of the percent difference of the determined concentrations from the nominal value and expressed as %RE. The deviations from theoretical values were ≤ 3% at all concentration levels assayed for each standard sample. The within - day precision, defined as the mean of the daily coefficients of variations at each concentration (n=5), ranged from 0.39% to 3.03%. The between - day precision, expressed as the coefficient of variations of the pooled four day data at each concentration (n=20), was in the range of 1.06% to 2.90%.

The detection limit of HPLC system was established by injecting decreasing amounts of spiked deproteinized serum onto the column. Limit of detection (LOD)was estimated directly by analysis of the method's peak to peak baseline noise and calculated using equation based on IUPAC model, proposed by Foley and Dorsey,[17] including standard deviation of blank signal and slope sensitivity. At a signal to noise ratio 3 : 1 it was 0.200 ng on column. Limit of quantitation (LOQ) was estabilished to be 0.625 ng injected onto the column, determined with acceptable accuracy and precision. This value, corresponded to a concentration of 0.125 μg/mL of the standard sample, included in the calibration curve as a lowest concentration level. The limit of detection can be affected by many variables, including the age and condition of the HPLC column and the variability associated with the sample preparation and dilution.

Table 1

Precision and Accuracy of the Assay Method for Determination of AmB

		Nominal Concentrations (μg/mL)						
		0.125	**0.250**	**0.500**	**1,000**	**5,000**	**10,000**	**15,000**
Day 1[1]	Mean	0.1287	0.2455	0.5116	1.0278	4.9487	9.9890	15.0219
	S D	0.0039	0.0048	0.0039	0.0040	0.1123	0.1408	0.1517
	C V (%)	3.03	2.00	1.00	0.39	2.27	1.41	1.01
	R E (%)	2.95	-1.80	2.32	2.78	-1.03	0.54	-0.15
Day 2[1]	Mean	0.1257	0.2472	0.5118	1.0240	4.9476	10.0106	15.0082
	S D	0.0027	0.0018	0.0029	0.0044	0.0831	0.1886	0.1591
	C V (%)	2.17	0.74	1.00	1.95	1.68	1.90	1.06
	R E (%)	0.56	-1.12	2.36	2.40	-1.05	0.11	0.05
Day 3[1]	Mean	0.1280	0.2456	0.5099	0.9940	5.0102	9.9438	15.0337
	S D	0.0028	0.0060	0.0029	0.0190	0.0912	0.1939	0.1308
	C V (%)	2.22	2.44	0.74	1.95	1.82	1.95	0.87
	R E (%)	2.40	-1.76	1.98	-0.90	0.20	-0.56	0.22
Day 4[1]	Mean	0.1277	0.2480	0.5126	1.0161	4.9401	9.9440	15.0210
	S D	0.0026	0.0032	0.0101	0.0040	0.0588	0.0967	0.1697
	C V (%)	2.07	2.49	2.59	0.39	1.19	0.98	1.14
	R E (%)	2.16	-0.80	2.52	1.61	-1.80	-0.56	0.14
Overall[2]	Mean	0.1275	0.2466	0.5115	1.0155	4.9616	9.9718	15.0212
	S D	0.0037	0.0055	0.0088	0.0115	0.0834	0.1881	0.1598
	C V (%)	2.90	2.23	1.72	1.62	1.72	1.90	1.06
	R E (%)	2.00	-1.36	-2.34	1.55	-0.77	-0.28	0.14

[1]n = 5 determinations.
[2]n = 20 determinations.
Precision expressed as %CV.
Accuracy expressed as %RE.

Application of the Assay Method

The analytical procedures described above have been successfully applied to determine AmB concentrations in samples collected over the period of 72 h from four dogs treated intravenously with liposomal amphotericin B (5 mg/kg

Table 2

Serum Amphotericin-B Concentrations of the Four Dogs Treated by Infusion of AmBisome (5 mg/kg)

Sample Time Post-Dose (Hours)	Concentration (μg/mL) Mean ± SD	Range
0.08	12.20 ± 15.40	3-22 - 29.99
0.33	17.58 ± 6.00	13.80 - 24.50
0.66	24.56 ± 6.43	17.24 - 29.34
1	37.81 ± 16.30	22.10 - 54.65
2	24.13 ± 13.01	9.69 - 34.95
4	19.14 ± 10.69	7.27 - 27.99
8	13.02 ± 7.09	5.23 - 19.10
12	9.79 ± 5.10	3.91 - 13.05
24	5.14 ± 2.71	2.10 - 7.28
48	1.52 ± 0.33	1.14 - 1.76
72	1.09 ± 0.16	1.07 - 1.10

body weight). Serum analysis (Table 2) showed that the concentration of the drug reached maximum value of 54.65 μg/mL 1h after the infusion, thereafter the concentration of drug decreased to the minimum values, but still present at a concentration above 1 μg/mL three days after the infusion.

CONCLUSION

Results, that have been obtained using reversed phase liquid chromatography, provides a fast method of determination and plasma-level monitoring of amphotericin B. High efficiency, short column containing small particle size (1.5μm), reduced analysis time, and solvent consumption and increased sensitivity of the method described, provided the high resolution separation and determination of amphotericin B, using little volume of sample. The lower detection limit was 0.125 μg/mL with 5 μL of sample, which, represents an improvement over the detection limits by previous methods. The sensitivity, specificity, accuracy, and reproducibility of this assay method permits fast analysis of large number of samples obtained from the pharmacokinetic studies.

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