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High-performance liquid chromatographic analysis of amphotericin B in plasma, blood, urine and tissues for pharmacokinetic and tissue distribution studies☆

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ABSTRACT

A sensitive and reproducible high-performance liquid chromatographic method was developed to assay ampherotericin B in plasma, blood, urine and various tissue samples. Amphotericin B was isolated from each sample matrix by solid-phase extraction (Bond-Elut). The eluate from Bond-Elut containing amphotericin B was injected onto a reversed-phase C_{18} column (Waters, μBondpak, 10 μm, 300 mm × 3.9 mm I.D.) with a mobile phase of 45% acetonitrile in 2.5 m*M* Na_2EDTA at 1 ml/min. Detection of amphotericin B was by ultraviolet absorption at 382 nm. Blood and tissues were homogenized and extracted with methanol prior to Bond-Elut extraction. The extraction efficiencies of amphotericin B from plasma, blood and tissues were approximately 90, 70 and 75%, respectively. The sensitivity of the assay was ≤5 ng/ml for plasma, ≤25 ng/ml for blood, 2.5 ng/ml for urine and 50 ng/g for tissues. The linearity of the assay method was up to 2.5 μg/ml for plasma, 5 μg/ml for blood, 500 ng/ml for urine and 500 μg/g for tissues. The assay was reproducible with an intra-day coefficient of variation (C.V., $n = 3$) of <5% in general for plasma, blood and tissues. The inter-day C.V. of the assay was <5% for plasma ($n = 5$), <10% for blood ($n = 4$) and <5% for tissues ($n = 3$). The overall variability in the urine assay was generally <10%. This method has demonstrated significant improvement in the sensitivity and reproducibility in assaying amphotericin B in plasma and especially in blood, urine and tissues. We have employed this assay to compare the pharmacokinetic and tissue distribution profiles of amphotericin B in rats and dogs following administration of Fungizone and ABCD (amphotericin B–cholesteryl sulfate colloidal dispersion), a lipid-based dosage form. In addition, the assay method for plasma and urine samples can also be applied to pharmacokinetics studies of amphotericin B in man.

INTRODUCTION

Amphotericin B is a polyene antifungal agent which, despite its nephrotoxicity, remains the drug of choice for the treatment of invasive and disseminated fungal infections. Recently, several lipid-based dosage forms of amphotericin B have been developed to improve its therapeutic index by altering its plasma and tissue distribution profiles [1–5]. Preclinical and clinical studies have

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demonstrated that lipid-based formulations of amphotericin B retain antifungal efficacy and may exhibit significantly altered pharmacokinetics, tissue distribution and toxicity profiles when compared to the conventional formulation of amphotericin B [6–9]. In order to characterize and compare the pharmacokinetics and tissue distribution of amphotericin B when it is administered in different dosage forms (conventional deoxycholate-solubilized amphotericin B or newly developed lipid-based formulations of amphotericin B), a sensitive, specific, accurate and reproducible analytical method is necessary for the measurements of amphotericin B concentrations in plasma, blood, urine and various tissues samples.

Several analytical methods have been published in the literature for the determination of amphotericin B concentrations in plasma or serum [10–14]. These methods either showed potential interferences by endogenous compounds or showed low extraction efficiency (70–80% for plasma) or inadequate sensitivity for their application in pharmacokinetic and tissue distribution studies. One method [10] utilized solid-phase extraction; however, an internal standard (N-acetylamphotericin B) that is not readily available was required to improve the specificity and reproducibility. Some articles have described procedures for analyzing tissue concentrations of amphotericin B by extraction of tissue homogenates with methanol and then direct injection of the methanol extract onto the high-performance liquid chromatography (HPLC) column [3,14,15]. Attempts to utilize the published methods to determine amphotericin B concentrations in blood, liver, kidney and lungs were not successful due to endogenous interferences, low extraction efficiency or inadequate sensitivity and reproducibility which limited their application in our comparative pharmacokinetic studies of amphotericin B.

In this study, we have developed a sensitive, specific, accurate and reproducible analytical method which can be applied universally for the determination of amphotericin B concentrations in plasma, blood, urine and various tissue samples (liver, kidney, spleen, lungs, heart, brain and skeletal muscles). The solid-phase extraction procedure applied in this study was simple, efficient and reproducible which permitted the development of the assay without the use of an internal standard. This sample clean-up procedure was necessary to eliminate the endogenous interfering compounds in the sample types analyzed. A stability study of amphotericin B in solutions, plasma and liver at various temperatures showed that amphotericin B is stable in these biological matrices when stored at −20°C. This method has been successfully employed for the analysis of amphotericin B concentrations in various biological samples obtained from preclinical pharmacokinetic and tissue distribution studies in both rats and dogs.

EXPERIMENTAL

Chemicals and materials

Authentic standard of amphotericin B (95+%) for this assay was obtained from Liposome Technology (Menlo Park, CA, USA). Other chemicals, reagents and materials used in the analytical procedures included sodium phosphate monobasic (NaH_2PO_4, analytical grade), sodium hydroxide, Na_2EDTA (dihydrate, Sigma, St. Louis, MO, USA), methanol (HPLC grade), acetonitrile (HPLC grade), dimethylsulfoxide (DMSO, reagent grade), and Bond-Elut (C_{18}, 1 ml, 100 mg, Analytichem International, Harbor City, CA, USA). Blank rat plasma, blood, liver, kidney, spleen, heart, lungs, brain and muscle tissues for constructing standard curves were supplied by Charles River (Boston, MA, USA). Blank rat urine was supplied by Liposome Technology.

Standard solutions and samples

Stock solutions of amphotericin B were prepared by dissolving amphotericin B authentic standard in a mixture of DMSO and methanol (1:1, v/v). Serial dilutions of the stock solution were made using a solvent of methanol–acetonitrile–1 m*M* Na_2EDTA (50:40:10, v/v). Standard curve samples of the various biological

fluids or tissues were freshly prepared by spiking aliquots of the standard solutions of amphotericin B into the blank samples (0.5 ml for each plasma or blood sample, 0.3 to 0.5 g for each tissue sample and 2 ml for each urine sample). The concentration ranges for the standard curve samples were approximately 5 to 2500 ng/ml for plasma, 50 to 5000 ng/ml for blood, 50 ng to 500 μg/g for tissues and 2.5 to 500 ng/ml for urine. The spiked samples were incubated at 37°C for 5–10 min prior to extraction and isolation.

Extraction and isolation procedures

Extraction and isolation of amphotericin B from the various biological fluids and tissues was accomplished using Bond-Elut cartridges connected to a vacuum extraction manifold. To increase the loading volume, the cartridge was connected to a 5-ml syringe using an interlok connector. The packing material in the cartridge was conditioned with methanol (1–2 ml), water (1–2 ml) and then 10 m*M* phosphate buffer, pH 7.4 (3 ml) before loading the samples or sample extracts. Plasma and urine samples were loaded directly onto the Bond-Elut without further processing, while blood and tissue samples were homogenized and extracted with methanol and the methanolic extracts loaded onto the Bond-Elut cartridges for isolation of amphotericin B. Blood (0.5 ml each) or tissue samples (0.3 to 0.5 g each) were added to 0.5 ml of 10 m*M* phosphate buffer (pH 7.4) and then homogenized using a Polytron (Brinkmann Instruments, Westbury, NY, USA) homogenizer for approximately 5–10 s. The sample homogenates containing amphotericin B were then extracted with 4 ml of methanol by vigorous vortex-mixing for 30 s and centrifuged at 2000 *g* for 10 min. The supernatant of the methanolic extract was then loaded onto the Bond-Elut cartridge.

Aliquots of the samples (0.5 ml of plasma or 1–2 ml of urine) or sample extracts (0.5–2 ml of methanolic extract of blood or tissues) were pipetted into the 5-ml syringe containing 4 ml of 10 m*M* phosphate buffer, pH 7.4. The sample solutions were then eluted through the cartridges by applying gentle vacuum to produce a flow-rate of < 1 ml/min. After the sample had completely passed through the packing materials in the cartridge, the cartridge was then washed with 3 ml of methanol–10 m*M* phosphate buffer, pH 7.4 (40:60, v/v). Any residual liquid remaining in the packing materials was further removed by centrifugation at 2000 *g* for 2–3 min. Amphotericin B retained on the Bond-Elut cartridge was then eluted using 0.75 ml (for plasma) or 1 ml (for other sample types) of acetonitrile–2.5 m*M* Na_2EDTA (60:40, v/v for plasma, 50:50 for other sample types). Finally, centrifugation was applied to completely remove the residual liquid containing amphotericin B in the cartridge and 100-μl aliquots of the eluate were injected onto the HPLC system for quantification.

Chromatography and quantification

The HPLC system used for the analysis of all the sample types included an LKB/Pharmacia Model 2150 HPLC pump (LKB/Pharmacia, Piscataway, NJ, USA) connected to a Bio-Rad Model AS-100 autosampler (Bio-Rad, Richmond, CA, USA) and a Waters μ-Bondapak HPLC column (C_{18}, 10 μm, 30 cm × 3.9 mm I.D., Waters Assoc., Milford, MA, USA) eluted with a mobile phase of acetonitrile–2.5 m*M* Na_2EDTA (45:55, v/v) at a flow-rate of 1 ml/min under ambient temperature. Detection of amphotericin B was accomplished by a Hewlett Packard Model 1050 programmable variable-wavelength UV detector (Hewlett Packard, Avondale, PA, USA) at a wavelength of 382 nm. The peak heights of amphotericin B on the chromatograms were determined using a Hewlett Packard Model 3196A integrator. A weighted ($1/y$) least-squares linear regression analysis of the standard curves was performed using the PCNONLIN program (Statistical Consultants, Lexington, KY, USA) to obtain the slope and intercept values.

RESULTS AND DISCUSSION

Chromatography and standard curves

Under the chromatographic conditions described above, amphotericin B was eluted in ap-

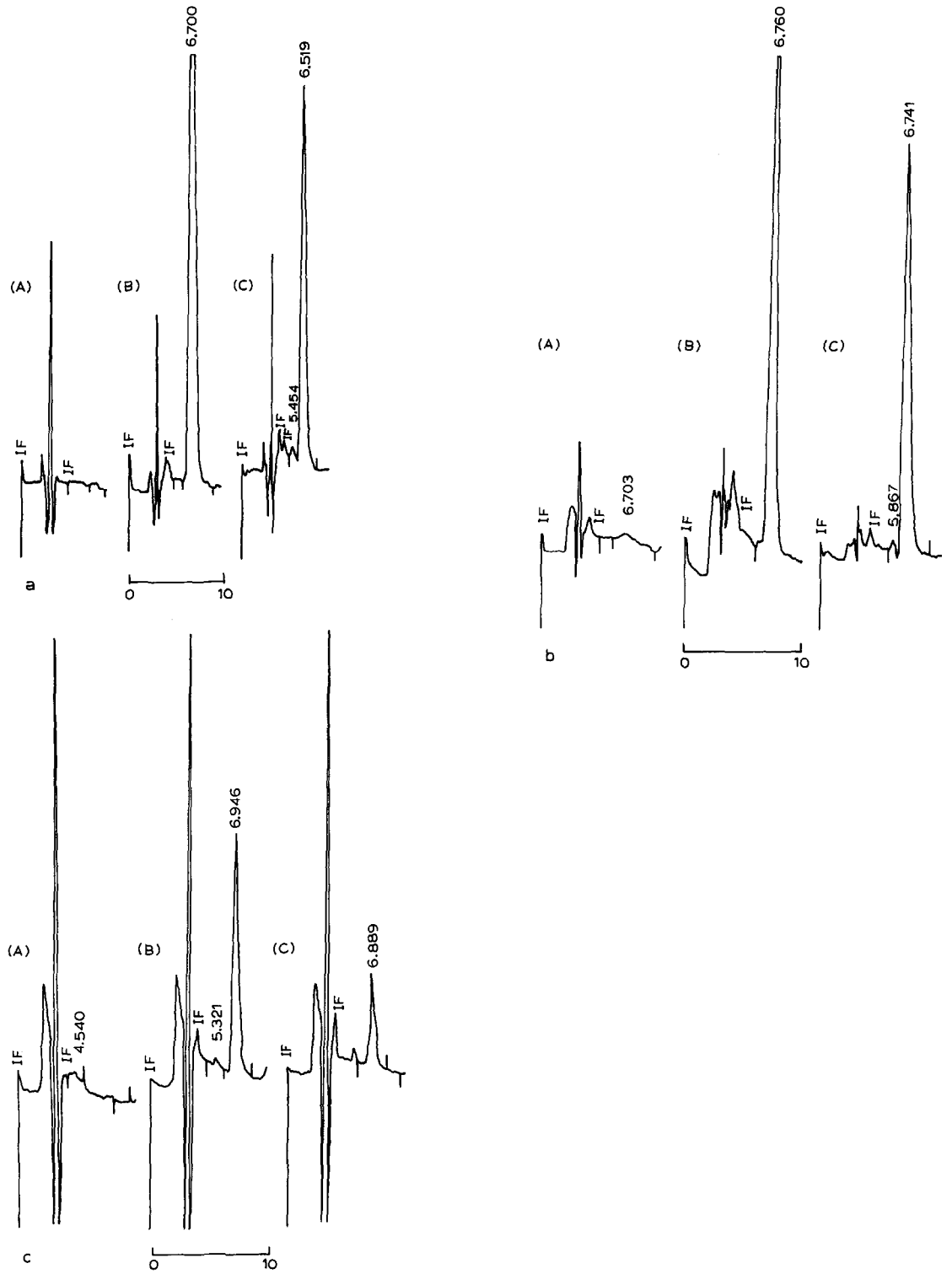

Fig. 1. (a) Typical chromatograms of amphotericin B in blank rat plasma (A), rat plasma spiked with 491 ng/ml amphotericin B (B) and rat plasma obtained after dosing of 5 mg/kg ABCD (C). (b) Typical chromatograms of amphotericin B in blank rat liver (A), rat liver spiked with 992 ng/g amphotericin B (B) and rat liver obtained after dosing of Fungizone (C). (c) Typical chromatograms of amphotericin B in blank rat urine (A), rat urine spiked with 50 ng/ml amphotericin B (B) and rat urine obtained after dosing of ABCD (C).

proximately 6–7 min. Typical chromatograms of blank rat samples (plasma, liver and urine), rat samples spiked with amphotericin B and rat samples obtained after intravenous administration of amphotericin B formulations are presented in Fig. 1a–c. Chromatograms of blood and other tissue samples were similar to those of liver samples. In addition, chromatograms of amphotericin B in plasma, urine and various tissue samples obtained from dogs were similar to those obtained in rats. Similar chromatograms were also obtained for human plasma and urine samples. The relatively short retention time of amphotericin B permitted the analysis of a large number of samples obtained from the pharmacokinetic studies. Minor adjustments of the composition of the mobile phase (percentage of acetonitrile) were made as necessary to maintain the retention time of amphotericin B at 6–7 min.

Standard curves for all sample types at the concentration ranges studied (see Experimental) were linear. The results of linear regression analysis show that the correlation coefficient of the standard curves for all sample types are ≥ 0.996.

Extraction effciency

The extraction efficiencies (recoveries) of amphotericin B from plasma, blood and liver were determined by spiking an equal amount of amphotericin B into the blank sample and a solution of acetonitrile–2.5 m*M* Na_2EDTA (60:40, v/v for plasma and 50:50, v/v for other sample types), respectively. Extraction efficiencies at three concentration levels were studied in triplicate for each sample type. The peak heights of amphotericin B on the chromatograms obtained from the extracted sample and the non-extracted sample (amphotericin B in solution) were then compared to determine the extraction efficiency. Extraction efficiency of amphotericin B averaged 92% from plasma at concentrations of 25–2500 ng/ml, 69% from blood at concentrations of 100–5000 ng/ml and 76% from liver at concentrations of 200–10 000 ng/g of tissue. Extraction efficiencies of amphotericin B from other tissue samples were similar to that of liver samples as shown by similar values of the slope for the standard curves between all tissue samples. The relatively high extraction efficiencies of amphotericin B from plasma (~90%) and other samples (~70% for blood and ~75% for liver) using this assay method are the basis for the improved sensitivity and reproducibility over other assay methods [3,15].

Linearity of the assay

Based on the results obtained from the standard curve samples, the assay method has a linearity up to 2500 ng/ml for amphotericin B in plasma, up to 5000 ng/ml for amphotericin B in blood, up to 500 ng/ml for amphotericin B in urine and up to 500 μg/g tissue for amphotericin B in liver and other tissue samples which included kidney, lungs, spleen, heart, brain and skeletal muscles. These levels were sufficiently high for the determinations of amphotericin B concentrations in the various biological samples obtained from rats and dogs following single and multiple intravenous administration of amphotericin B formulations [16,17].

Sensitivity of the assay

Based on the heights of amphotericin B peaks on the chromatograms obtained from the standard curve samples at the lowest concentration level and a 10:1 signal-to-noise ratio, the detection limit of the assay method was determined to be $\leq$ 5 ng/ml for plasma, $\leq$ 25 ng/ml for blood, 2.5 ng/ml for urine and 50 ng/g of tissue for liver and other tissue samples. In addition, the accuracy and reproducibility of the assay for the lowest concentration value of the standard curve for each sample type have been validated ($<$ 5% deviation from theoretical values; $<$ 10% coefficient of variation, C.V.). Lower detection limits could be achieved by increasing the HPLC injection volume or by increasing the volume of sample extracts loaded onto the Bond-Elut cartridges.

Selectivity of the assay

As shown by the chromatograms of blank plasma, blood, urine, liver and other tissue samples collected from rats without receiving any amphotericin B formulations (Fig. 1), there are

TABLE I

ACCURACY OF THE ASSAY METHOD FOR DETERMINING AMPHOTERICIN B CONCENTRATIONS IN PLASMA AND LIVER SAMPLES

Day No.	Plasma samples					
	Low (24.7 ng/ml)		Medium (246.8 ng/ml)		High (2467.7 ng/ml)	
	Concentration found (ng/ml)	Deviation (%)	Concentration found (ng/ml)	Deviation (%)	Concentration found (ng/ml)	Deviation (%)
1	24.1	−2.4	242.7	−1.7	2402.2	−2.7
2	25.3	+2.4	243.6	−1.3	2407.9	−2.4
3	24.9	+0.8	253.0	+2.5	2571.6	+4.2
Mean	24.8	+0.4	246.4	−0.2	2460.6	−0.3

Day No.	Liver samples					
	Low (99.2 ng)		Medium (992 ng)		High (4960 ng)	
	Amount found (ng)	Deviation (%)	Amount found (ng)	Deviation (%)	Amount found (ng)	Deviation (%)
1	108.6	+9.5	1039	+4.7	4757	−4.1
2	98.6	−0.6	986	−0.6	5014	+1.1
3	104.4	+5.3	1001	+0.9		
Mean	103.9	+4.7	1008.7	+1.7	4886	−1.5

no interfering peaks present at the retention time for the amphotericin B peak on the chromatograms. A minor peak with a peak height less than one tenth of that for the lowest standard sample occurred occassionally in some blank tissue samples at time shortly before the amphotericin B peak. This peak represents the background noise level and thus does not interfere with the accu-

TABLE II

INTRA-DAY VARIABILITIES OF THE ASSAY METHOD FOR DETERMINING AMPHOTERICIN B CONCENTRATIONS IN PLASMA AND LIVER SAMPLES

	Plasma (n = 3)		Liver (n = 3)	
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Amount found (mean ± S.D.) (ng/g)	C.V. (%)
Low	24.9 ± 1.1	4.5	100 ± 4.3	4.3
Medium	242.9 ± 4.8	2.0	982 ± 17.6	1.8
High	2393 ± 11.0	0.5	9030 ± 411	4.6

racy or reproducibility of the assay. The use of UV detection at a wavelength of 382 nm excludes the detection of most of other therapeutic agents. The possible endogenous interferences are bilirubin and hemoglobin [10,13] which were frequently present in the methanol extract of blood, liver and lung samples and were eliminated during the solid-phase extraction procedure.

Accuracy of the assay

The accuracy of the assay method in determining amphotericin B concentrations in spiked plasma and liver samples is presented in Table I. The accuracy of the assay method was also determined for blood and urine samples which were similar to those for plasma and liver samples. The deviation from theoretical values is generally $< 5\%$ at all concentration levels assayed for each sample type. Since the extraction efficiencies of amphotericin B from other tissue samples were similar to that from the liver, the accuracy of the assay method for other tissue samples was not determined.

Intra-day variability of the assay

The intra-day variabilities of the assay method for plasma and liver samples are shown in Table II. In general, the intra-day variability as determined by the C.V. in repeated analyses of the same sample on the same day is less than 5% for plasma and liver samples over a wide concentration range. These data indicate that the assay method is reproducible within the same assay run.

Inter-day variability of the assay

The inter-day variabilities of the assay method as determined for the quality control samples over a one-month period are presented in Table III for plasma and liver samples. The inter-day variability data for blood samples were also obtained and found to be similar to those for plasma and liver samples. The quality control samples at three concentration levels for plasma, blood and liver samples were prepared as a single batch on the same day and then aliquots of each sample were stored at $-20°C$ until analysis on different days. The inter-day variabilities of the assay method as determined by C.V. in repeated analyses of an aliquot of the same sample over an extended period are generally less than 5% for the various types of samples over a wide concentration range. Additional data also showed that C.V.s for the analysis of plasma quality control samples over a five-month period were less than 10% at the three concentration levels. The slopes of the standard curves of the various sample types also did not change significantly over an extended period with a variability (C.V.) generally less than 10%. These data indicate that the assay method for the various sample types is reproducible over an extended period. The combined intra- and inter-day variability of the urine assay is 10% for the lowest concentration assayed and is 5% for the highest concentrations assayed.

Stability of amphotericin B

The effects of storage conditions and temper-

TABLE III

INTER-DAY VARIABILITIES OF THE ASSAY METHOD FOR DETERMINING AMPHOTERICIN B CONCENTRATIONS IN PLASMA AND LIVER QUALITY CONTROL SAMPLES OVER A ONE-MONTH PERIOD

	Plasma (n = 5)		Liver (n = 3)	
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Amount found (mean ± S.D.) (ng/g)	C.V. (%)
Low	25.4 ± 0.9	3.5	103.9 ± 5.0	4.8
Medium	247.8 ± 6.8	2.7	1009 ± 27	2.7
High	2455 ± 74	3.0	4967 ± 191	3.9

ature on the stability of amphotericin B in solutions, plasma and liver homogenates were determined under various temperatures over different time periods. Three amphotericin B stock solutions prepared over a period of eighteen months were assayed for amphotericin B concentrations in comparison to a freshly prepared stock solution. All stock solutions were diluted with methanol–acetonitrile–1 m*M* Na_2EDTA (50:40:10, v/v) and the freshly diluted solutions were injected directly onto the HPLC system for comparison of amphotericin B peak heights. The amphotericin B concentrations determined for the stock solutions stored at −20°C over various time periods up to eighteen months were almost unchanged with little or no degradation (0% loss at nine months, 3.7% loss at twelve months and 1.2% loss at eighteen months). These data indicate that amphotericin B in DMSO–methanol (1:1, v/v) stored at −20°C is stable up to a period of eighteen months. These results are encouraging as compared to a previous report which showed greater than 60% of amphotericin B in 100% DMSO degraded after storage at −20°C for six months [18].

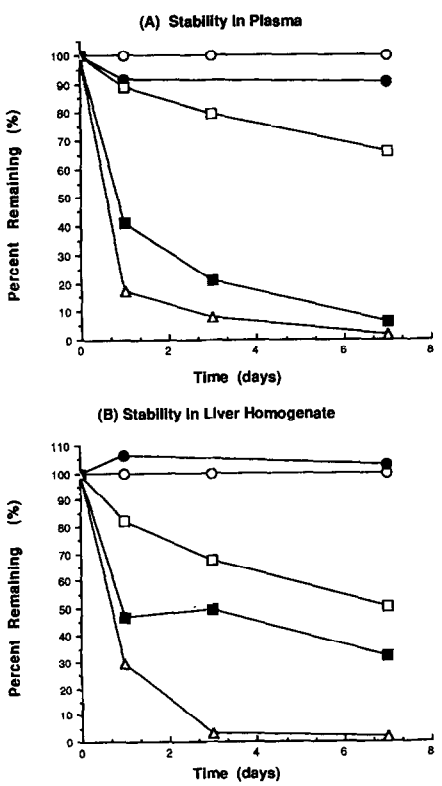

Fig. 2. Amphotericin B stability in rat plasma (A) and rat liver homogenate (B) at various temperatures. (○) Fresh samples; (●) samples stored at −20°C; (□) samples stored at 4°C; (■) samples stored at 25°C; (△) samples stored at 37°C.

Plasma samples spiked with 2500 ng/ml amphotericin B and liver homogenates spiked with 10 μg amphotericin B per g of tissue were stored at −20, 4, 25 and 37°C over time periods of up to seven days. Concentrations of amphotericin B in these samples were determined on days 0, 1, 3 and 7, respectively, in comparison to the freshly spiked samples at the same concentration level. Fig. 2 shows the percentage amphotericin B remaining in the plasma and liver samples (n = 3), respectively, stored under the various temperatures over different time periods. Amphotericin B concentrations in plasma and liver homogenates stored at −20°C showed almost no degradation for at least seven days, indicating that amphotericin B in plasma or liver sample is stable at −20°C. A previous study showed that amphotericin B is stable in frozen serum (−20°C) for up to six months [18]. Degradation of amphotericin B occurs in plasma or liver samples when stored at 4°C or higher temperatures and the rate of degradation is temperature-dependent. The degradation of amphotericin B in plasma and liver samples is primarily due to autoxidation of amphotericin B at the polyene moiety by light or heat-stimulated free radical mechanism [19,20]. The present study indicates that control of temperature during sample storage and work up is important to ensure the stability of amphotericin B in various biological matrices. A previous study documented amphotericin B decay in a liquid medium (L-asparagine, dextrose in water) at pH 7.4 under 37°C with a decay half-life of 4–5 h [20]. In our study, the degradation half-life of amphotericin B in plasma or liver homogenates at 37°C was approximately 16 h. The differences between the two studies may be due to the different media and/or assay procedures used. The half-lives of amphotericin B degradation in plasma and liver

at 4 and 25°C were approximately > seven days and one day, respectively.

Assay variability in liver samples from different parts of the liver

The assay variability in determining amphotericin B concentrations in liver samples obtained from three different parts of the same liver was examined. The stability of amphotericin B in frozen whole liver was also determined by assaying the same liver on different days over a period of seven days. The variability in determining amphotericin B concentrations in liver samples obtained from different parts of the liver was generally less than 10% over a wide concentration range (6.8% at 1.95 μg/g of tissue, 5.3% at 40.7 μg/g of tissue and 9.0% at 124.8 μg/g of tissue). The variability in determining amphotericin B concentration in the same liver over a one-week period was 10% (n = 3). These results show that the assay method is reproducible from day to day in determining amphotericin B concentrations throughout the entire liver and that amphotericin B appears to be homogeneously distributed throughout the liver. In addition, amphotericin B is stable for at least one week in rat liver kept frozen at −20°C.

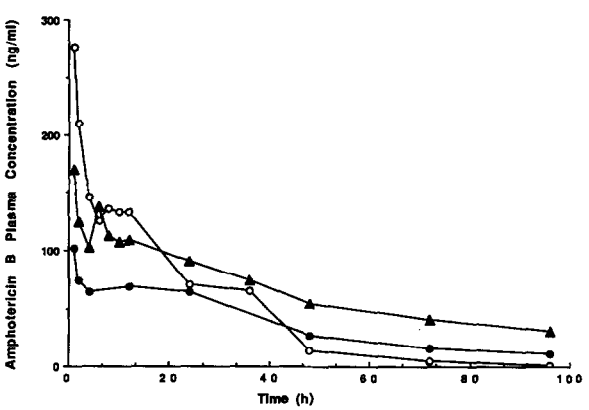

Fig. 3. Plasma concentrations of amphotericin B in rats following a single intravenous injection of fungizone or ABCD. (○) 1 mg/kg Fungizone; (●) 1 mg/kg ABCD; (▲) 5 mg/kg ABCD. Concentration *versus* time curve for each treatment was obtained by pooling data from four groups of rats with each group containing five rats and three serial blood samples taken from each rat at different time points.

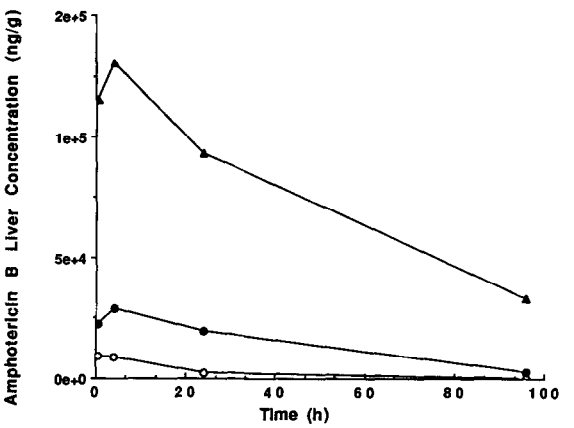

Fig. 4. Liver concentrations of amphotericin B in rats following a single intravenous injection of fungizone or ABCD. (○) 1 mg/kg Fungizone; (●) 1 mg/kg ABCD; (▲) 5 mg/kg ABCD. Concentration at each time point represents the mean value of five rats.

Application of the assay method

The analytical procedures described above have been successfully applied to determine amphotericin B concentrations in the various sample types collected from rats and dogs after single or multiple intravenous administration of two different amphotericin B formulations, micellar amphotericin B deoxycholate (Fungizone, E. R. Squibb & Sons, Princeton, NJ, USA) and amphotericin B–cholesteryl sulfate colloidal dispersion (ABCD, Liposome Technology), a lipid-based formulation [16,17]. Figs. 3 and 4 show the plasma and liver concentration *versus* time curves of amphotericin B in rats following a single intravenous administration of 1 mg/kg Fungizone, 1 mg/kg ABCD and 5 mg/kg ABCD, respectively. The sensitivity, specificity, accuracy and reproducibility of this assay method facilitate thorough studies of the pharmacokinetics and tissue distribution of amphotericin B in animals, especially for the comparison of the pharmacokinetic profiles of amphotericin B following administration of different formulations of amphotericin B. Most importantly, this assay method has demonstrated significant improvement in the extraction efficiency, sensitivity and reproducibility in analyzing amphotericin B in plasma, blood, urine and various tissue samples over other existing assay methods.

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