Journal of Chromatography, 274 (1983) 271—279
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1614

AN INTERNALLY-STANDARDIZED ASSAY FOR AMPHOTERICIN B IN TISSUES AND PLASMA

JAMES W. MAYHEW*, CYNTHIA FIORE, THOMAS MURRAY and MICHAEL BARZA

Infectious Disease Service, New England Medical Center, 171 Harrison Avenue, Boston, MA 02111 (U.S.A.)

(First received September 20th, 1982; revised manuscript received December 23rd, 1982)

SUMMARY

A high-performance liquid chromatographic (HPLC) method with *p*-nitrophenol as internal standard is described for the rapid analysis of amphotericin B recovered by methanolic extraction from tissues and plasma. Programmed, gradient elution of the ODS column was used with detection by tungsten light at 388 nm. Standard curves were derived based on the peak height ratios. The lowest reproducible limit of the assay was 0.04 μg/ml with plasma. The extraction and chromatographic procedures recovered 53—71% of the amphotericin B from each of these sources. The coefficient of variation of the recovery ratios was less than 18% from plasma over a range of concentrations of amphotericin B from 0.08 to 10.0 μg/ml. Recovery from tissues, studied over a narrower concentration range, showed a similar degree of precision. Variations in precolumns apparently resulting in selective binding of the amphotericin B were found to have a systematic but important influence on recovery efficiency. No substances were detected which interfered with the assay procedures as described. By incorporating an internal standard we have enhanced the reliability and flexibility of the HPLC assay for amphotericin B especially for assay of tissues.

INTRODUCTION

Amphotericin B, a non-aromatic heptaene compound, is the drug of choice for most systemic fungal infections. Its antimycotic properties are related to its ability to form strong hydrophobic bonds to sterols, especially ergosterol, in the membranes of fungal cells [1, 2]. Binding to sterols in the membranes of animal cells may contribute to the prolonged tissue retention of the drug [3]. Amphotericin B has been detected in the serum and bile of dogs for more than 7 days, in urine for more than 20 days, and in kidney tissue for 50 days, after a single dose [4]. However, many of the pharmacological characteristics of this compound remain poorly understood [5].

0378-4347/83/$03.00 © 1983 Elsevier Science Publishers B.V.

The major limitation to the use of amphotericin B is its toxicity. Most patients treated with this agent develop adverse reactions, particularly impairment of renal function [6–8]. Although toxicity is generally dose-dependent, there is considerable interindividual variability. It has been difficult to establish a clear relation between blood levels of amphotericin B and its efficacy or toxicity.

Many of the difficulties encountered in studying the pharmacology and toxicity of amphotericin B arise because of problems in assaying the compound. Microbiological assays are satisfactory for many purposes [9], but generally lack the precision, sensitivity and speed of chromatographic procedures. Direct spectrophotometric assay at 405 nm may be affected by products of hemolysis or by other drugs [10]. Both types of assay are difficult to apply to tissue specimens.

High-performance liquid chromatographic (HPLC) procedures have been used to determine serum or plasma antibiotic levels in the microgram to nanogram range which would be crucial for assays of the drug in whole tissues [10–13]. However, methods described so far for amphotericin B have failed to incorporate an internal standard [10, 14]. The internal standard corrects for inadvertent changes in drug concentration incurred during extraction, dilution or concentration of plasma or tissue homogenates. We report here a sensitive and precise HPLC assay for amphotericin B incorporating an internal standard.

MATERIALS AND METHODS

Apparatus and reagents

An Altex (Berkeley, CA, U.S.A.) high-performance liquid chromatograph was used. The instrument was equipped with two Model 110A pumps, a Rheodyne Model 7125 injector (50-μl load loop), a Model 420 Microprocessor System Controller, and a reversed-phase Ultrasphere-ODS column (15 cm × 4.6 mm I.D.; 5 μm average particle size). One chromatographic column was used for all experiments. A 3.0-cm C_{18} precolumn was used (Brownlee Labs., Santa Clara, CA, U.S.A.). The precolumns were either RP-18 LiChrosorb (10 μm) or RP-18 Spheri-5 (5 μm). Eluents were monitored by absorbance of tungsten light at 388 nm on a 0.05 a.u.f.s. scale using a Hitachi Model 100-30 spectrophotometer. Flow-rate was maintained at 1.0 ml/min. HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethylene dinitrilotetraacetic acid disodium salt (EDTA) and *p*-nitrophenol (PNP, the internal standard) were from Sigma (St. Louis, MO, U.S.A.). Amphotericin B type 1 reference powder was generously supplied by E.R. Squibb (Princeton, NJ, U.S.A.). The drug was kept in stock at a concentration of 2500 μg per ml of methanol—dimethyl sulfoxide (1:1) and stored at −20°C. *p*-Nitrophenol was stored at 5°C as 3.0 mg per ml methanol. Two separate pools of the drug and of the internal standard were maintained. These stock solutions were alternately sampled to determine standard values. New pools were introduced every three to four weeks and verified against the old pools for their concentration of amphotericin B.

Chromatography

The mobile phase components consisted of 50% methanol in 0.01 *M* EDTA (phase A) and 50% acetonitrile in 0.01 *M* EDTA (phase B). For the programmed elution gradient, the system was maintained in phase A for 6 min, then shifted to phase B over a span of 1 min. After 15 min, the mobile phase was progressively shifted back to phase A over a span of 0.5 min. A new specimen could be introduced every 19 min.

The recovery value for amphotericin B from individual samples was calculated as the ratio of the peak height of the deflection produced by amphotericin B to that produced by PNP (internal standard, 150 μg). Calculations based on the ratios of areas under the curve were generally less precise than those based on peak height ratios. Standard curves were derived by linear regression analysis of specimens containing known concentrations of amphotericin B over a range of values.

Tissue extraction procedures (liver, kidney, spleen)

The recovery of amphotericin B was studied in samples to which known quantities of the drug were added before homogenization (spiked homogenates) or in which the drug was added to methanol extracts of tissue homogenates (spiked extracts). The former preparations were expected to simulate the tissues of treated animals; the latter were compared with these to determine whether there was a difference in recovery of drug attributable to the presence of whole tissue. In each instance, 150 μg PNP (50 μl of methanolic stock) were mixed and equilibrated with the tissues before homogenization.

Approximately 650 mg of kidney were used (range 620—770 mg), 500 mg of liver (range 420—650 mg) and 350 mg of spleen (range 250—490 mg). The extraction process was carried out by adding 1.5—2.0 ml of methanol to tissues which were then finely minced in a microhomogenizer cup (Sorvall, Norwalk, CT, U.S.A.). The cup was suspended in ice, and the mixture was homogenized at high speed for three 30-sec periods. The homogenate together with two methanol rinses (0.5—1.5 ml) of the cup was decanted into a 15-ml cork-stoppered Corex tube and heated (50°C) in a shaking water bath for 15 min. The extraction mixture was then centrifuged (12,000 *g* for 10 min) and the clear supernatant was collected. The pellet was reextracted and the clear supernatants were pooled. On occasion, it was necessary to clarify the extracts by further centrifugation or by filtration (0.5 μm Millex SR, Millipore, Bedford, MA, U.S.A.).

Plasma extraction procedures

A 150-μg amount of PNP (50 μl of methanolic stock) and 3.0 ml of methanol were added to 1.0 ml of plasma containing known quantities of amphotericin B. The opaque mixture was vigorously agitated and then centrifuged at 12,000 *g* for 10 min. The clear supernatant was collected for HPLC analysis.

RESULTS

Amphotericin B had a retention volume of 14.0 ml and PNP a retention volume of 5.4 ml in the chromatographic system described. The small amounts

of detectable background from tissue extracts and plasma or serum did not interfere with quantitation of the drug. Fig. 1 shows chromatograms of methanolic extracts of mouse liver homogenates (left) and human plasma (right) without amphotericin B. There were no significant chromatographic bands in the elution regions of the internal standard or of amphotericin B. The chromatograms which resulted from adding 5.0 μg of amphotericin B to 500 mg of mouse liver homogenate or 2.5 μg of amphotericin B to 1.0 ml of human plasma, together with PNP, are also depicted in Fig. 1 (lower panels). Similar results were obtained with spleen and kidney. The small chromatographic band eluting at 13.0—13.2 ml is recovered only in the presence of the drug. A similar

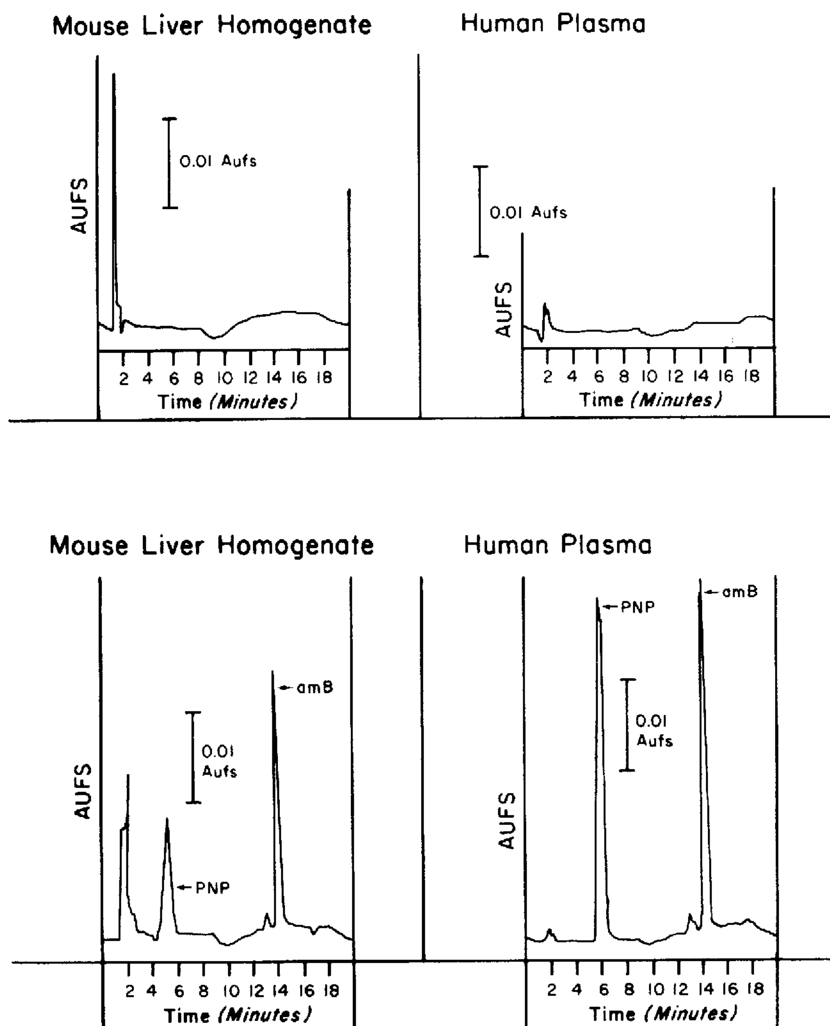

Fig. 1. Top panels: chromatograms of a methanolic extract of homogenized mouse liver and of human plasma; bottom panels: chromatograms of the same substances with amphotericin B (amB) and internal standard (*p*-nitrophenol, PNP) added before the extraction procedure. A.u.f.s. signifies absorbance units full scale.

band which may represent a separate component of amphotericin B has been previously reported [10, 14].

A primary source of analytical variation can be found in the chromatographic system itself. Changes in the slope of the ratio recovery curves can be linked to the state of the guard or precolumn (age, previous use, lot number). To verify this conclusion, a methanolic plasma extract (5 μg amphotericin B per ml plasma) supplemented with PNP was repeatedly chromatographed. The mean height ratios (amphotericin B:PNP) varied strikingly from column to column as shown in Table I. These discrepancies were generally statistically significant. For example, the values for column L_1 exceeded those for all other columns ($p < 0.01$ by unpaired t-test). In contrast, within-column variations were slight as shown by the small standard deviations. The absolute peak heights for the internal standard, PNP, were not significantly different among precolumns, varying from 25—30 mm in 24 tests. However, the peak heights for amphotericin B ranged from 18—83 mm, suggesting that the precolumn selectivity primarily affected the drug and not the internal standard. The precolumn was routinely used as a protective device; however, in the few instances in which it was omitted, height ratios near 3.0 were encountered. When the results for each precolumn are considered separately, the coefficients of variation (C.V.) were very small (Table I).

TABLE I

THE CHROMATOGRAPHIC RECOVERY OF AMPHOTERICIN B FROM PLASMA USING FIVE DIFFERENT PRECOLUMNS

Multiple aliquots from a single methanolic extract of human plasma containing 5 μg amphotericin B per ml were chromatographed for each precolumn.

Precolumn*	Mean recovery ratio ± S.D.**	C.V. (%)
L_1	2.56 ± 0.18 (8)	7.0
L_2	1.40 ± 0.04 (5)	3.0
L_3	1.90 ± 0.11 (5)	6.0
S_1	0.76 ± 0.06 (5)	7.0
S_2	2.80 (1)	

*L (LiChrosorb), S (Spherisorb); L_1, L_3, S_2 new precolumns with no or very limited use; L_2 and S_1 in use for approximately one month.
**Values are the mean ± standard deviation (S.D.) of the ratios of the peak height of amphotericin B:PNP. Values in parentheses indicate the number of trials.

In order to assure that the assay was functioning properly each day, an initial run was made using pools of methanolic standards (PNP + 5 μg amphotericin B in methanol) maintained at —20°C. When the elution characteristics were abnormal or peak height ratios of these standards departed by more than 2 S.D. from previous values, the equipment was rechecked for mechanical problems; however, such variations often indicated the need for replacement of the precolumn.

Estimates of the effect of the biological matrix upon recovery of amphotericin B were made by comparing the height ratios observed with methanol-diluted standards to those from drug-supplemented tissues (Table II) or plasma

TABLE II

THE RELATIVE EFFICIENCY OF AMPHOTERICIN B RECOVERY FROM MOUSE TISSUE HOMOGENATES

Amphotericin B (μg)	Mean recovery ratio		Relative recovery
	Tissue*	Methanol standard	(Mean tissue—mean methanol standard)
2.5	0.98 (9)	1.50 (6)	0.65
5.0	1.83 (9)	2.85 (6)	0.63
10.0	4.24 (7)	5.90 (5)	0.71

*Values are the mean of the peak height ratio of amphotericin B to that of PNP in separate analyses of five liver homogenates, two kidney homogenates, and two spleen homogenates. Each sample contained 350—650 mg of tissue and was supplemented with known quantities of amphotericin B and PNP. Values in parentheses are the number of separate preparations analyzed.

TABLE III

THE RELATIVE EFFICIENCY OF AMPHOTERICIN B RECOVERY FROM HUMAN PLASMA

Amphotericin B (μg)	Mean recovery ratio		Relative recovery
	Plasma*	Methanol standard	(Mean tissue—mean methanol standard)
2.5	0.93 (10)	1.70 (4)	0.53
5.0	1.99 (10)	3.30 (4)	0.61
10.0	4.61 (10)	7.50	0.61

*Values are the mean of the peak height ratio of amphotericin B to that of PNP in separate analyses of ten different human plasma pools supplemented with known quantities of amphotericin B and PNP. Values in parentheses are the number of separate preparations analyzed.

(Table III). There were no observable differences in the efficiency of recovery among liver, kidney, and spleen homogenates; therefore, the data for these tissues were pooled in Table II. The relative recovery of amphotericin B from tissue was 63—71% and from human plasma was 53—61% of the corresponding values for methanol standards. Recovery did not appear to be correlated with the absolute quantity of the drug. These data indicate that as much as 47% of amphotericin B is either not readily extractable from tissues and plasma or is in a bound or complexed form which does not fractionate efficiently in the chromatographic system. Only another 1—2% of the amphotericin B could be recovered by reextraction of pellets. The overall extraction efficiency of PNP was 85—90%. When amphotericin B was added to methanolic supernatants of tissue extracts, recovery ratios were statistically indistinguishable from those for methanolic standards. Thus, the interfering substances were either not methanol soluble or were unable to interact with amphotericin B in methanol.

Regression equations were calculated for the recovery of known quantities

of amphotericin B from the tissue homogenates. Two different precolumns, L_1 and L_2, were used. The recovery slope and the intercept for the L_1 set were 0.66 (r^2 = 0.99) and 0.075, respectively, and for the L_2 set were 0.44 (r^2 = 0.99) and −0.23, respectively. The relationship between the recovery ratio and the quantity of amphotericin B is linear. The coefficients of variation for the two sets of data ranged from 10—20%.

The precision of recovery of amphotericin B added to plasma at concentrations in the range 0.01—10.0 μg/ml is shown in Table IV. The minimum reproducible limit of the assay as described was 0.04 μg/ml. Lower values, 0.02 and 0.01 μg/ml appeared as barely discernible chromatographic bands which were too small for precise measurement. Within the range 0.04 to 5.0 μg/ml, the recovery slope (0.37; r^2 = 0.96) was linear. The coefficient of variation for

TABLE IV

THE PRECISION OF AMPHOTERICIN B RECOVERY FROM HUMAN PLASMA*

Amphotericin B (μg/ml)	Mean recovery ratio ± S.D.	C.V. (%)
0.01	0.010 ± 0.000 (5)	**
0.02	0.010 ± 0.000 (5)	**
0.04	0.015 ± 0.002 (6)	36.5
0.08	0.034 ± 0.003 (5)	16.1
0.16	0.058 ± 0.005 (4)	16.6
0.31	0.134 ± 0.008 (5)	12.5
0.63	0.255 ± 0.007 (4)	5.0
1.25	0.460 ± 0.031 (7)	17.9
2.50	0.93 ± 0.13 (10)	14.0
5.0	1.99 ± 0.16 (10)	8.0
10.0	4.61 ± 0.55 (10)	11.9

*See footnote for Table III.
**Peaks were too small for precise measurement.

TABLE V

AMPHOTERICIN B RECOVERY FROM THE LIVER AND KIDNEYS OF MICE

Mice were given a single i.v. dose of amphotericin B and were killed at the indicated time. The liver and kidneys were processed to determine drug concentrations.

i.v. Dose (mg/kg)	Time after injection (h)	Mouse No.	Liver (μg drug per g tissue)	Kidney (μg drug per g tissue)
1.5	1	0	14.58	3.48
		1	20.91	2.95
		2	14.15	0.65
1.5	42	6	10.03	1.42
		7	12.56	2.19
		8	13.30	2.70
2	72	12	14.39	2.52
		13	10.54	2.92
		14	36.43	5.38

the results ranged from 36.5% at 0.04 μg/ml to 5% at 0.625 μg/ml. Accordingly, the precision of drug recovery from plasma was very similar to that for tissues.

Nine mice were given single intravenous (i.v.) injections of amphotericin B in distilled water, after which drug levels in the liver and kidneys were determined by the chromatographic method (Table V). Although the three groups are not fully comparable because the dosages and timing of study were different, the values for each tissue within a group were fairly similar except for mouse 2 (low levels in kidney) and mouse 14 (high levels in both tissues). Hepatic levels were 3—20 fold greater than those recovered from the kidney.

DISCUSSION

Amphotericin B remains the most useful of agents for the therapy of most deep-seated fungal infections. However, its use is complicated by the almost universal development of toxic reactions, particularly those affecting the kidney. The pharmacokinetic behavior of amphotericin B is unusual. The drug is extensively bound to sterol-containing membranes and is excreted slowly and incompletely in the urine and bile [4, 15]. Investigations into the pharmacokinetic properties and mechanisms of toxicity of the compound have been hampered by lack of a simple, rapid and precise assay method capable of being applied to tissues and in the presence of other drugs.

A variety of microbiological assays have been described [16]. These are convenient for certain clinical purposes [9]. In comparison with the HPLC assay, however, bioassays are somewhat less precise, are difficult to standardize, and pose problems in interpretation especially at low drug concentrations [10]. Microbiological bioassays require 16—18 h of incubation. Natural antifungal activity in the blood of normal individuals may interfere with the assay as may other drugs given concomitantly. Direct spectrophotometric and microbiological assays may be difficult to interpret because of heme and other colored substances present in plasma and tissues [16].

The present study is the first report of an internally-standardized chromatographic assay for amphotericin B in tissues and plasma. The methanolic extraction used in our laboratory recovers 53—71% of the amphotericin B present in tissues and plasma. The data suggest that the avidity of binding to tissues and plasma are nearly equivalent.

The internal standard acts as a correction for procedural losses which might be incurred during extraction, dilution, filtration or chromatographic manipulations particularly when one is working with tissues. This has been especially helpful when concentration of the methanolic extract by evaporation is desirable in order to quantitate very low levels of drug in tissues. PNP, of course, is chemically very unlike amphotericin B. However, our efforts to find a useable congener of amphotericin B to serve as an internal standard were unsuccessful. N-acetylamphotericin B and the methyl ester of amphotericin B were initially considered; however, the former was difficult to acquire as a pure standardized powder and the latter had undesirable chromatographic elution characteristics (avid association with the RP column). PNP was an attractive choice because of its solubility and its chromatographic and light-

absorption properties. The linearity of the recovery curves over a wide range of concentrations and the low coefficients of variation at each concentration suggest that the extractability of amphotericin B and *p*-nitrophenol are similar under the circumstances of the assay. Although amphotericin B has a striking absorption peak at 405 nm, detection at 388 nm (visible) slightly enhanced the recovery precision for both compounds in these studies.

The data presented in Table I demonstrate a marked effect of the precolumn on the analytical yield. The recovery of PNP as determined by peak height measurement was unaffected. In contrast, the absolute recovery of amphotericin B was strikingly dependent upon the precolumn. Methanol-soluble substances from the biological matrix may bind avidly to certain precolumns and may in turn bind amphotericin B. The slow elution of such a complex or of the drug from the complex might not produce a discrete chromatographic band which could be distinguished from the background.

The lowest reproducible limit of the assay as described is 0.04 μg/ml (C.V. 36%). As in the case of most chromatographic assays, this one can be refocused to a lower range. Reduction in the quantity of PNP, concentration of methanolic extracts by evaporation, increase of the injection volume, and increase of the absorbance scale all may contribute to increased sensitivity of the assay. We have not fully explored the minimum concentration of drug in tissue at which the signal-to-noise ratio would limit sensitivity. Preliminary tests indicate the critical point to be less than 0.01 μg/ml plasma and 0.02 μg/g tissue. Even in tissue extracts concentrated 8-fold by evaporation, we have failed to find interfering chromatographic bands which elute in the region of PNP or amphotericin B.

REFERENCES

1 R.Y. Cartwright, J. Antimicrob. Chemother., 1 (1975) 141.
2 W.C. Chen, D-L Chou and D.S. Feingold, Antimicrob. Ag. Chemother., 13 (1978) 914.
3 J.E. Bennett, New Engl. J. Med., 290 (1974) 914.
4 P.C. Craven, T.M. Ludden, D.J. Drutz, W. Rogers, K.A. Baegele and H.B. Skrdlant, J. Infect. Dis., 140 (1979) 329.
5 G. Medoff and G.S. Kobayashi, New Engl. J. Med., 302 (1980) 145.
6 W.R. Butler, J.E. Bennett and D.W. Alling, Ann. Intern. Med., 61 (1964) 175.
7 R.P. Miller and J.H. Bates, Ann. Int. Med., 71 (1969) 1089.
8 J.L. Burgess and R. Birchall, Amer. J. Med., 53 (1972) 77.
9 R.B. Bannatyne, R. Cheung and E.R. Devlin, Antimicrob. Ag. Chemother., 11 (1977) 44.
10 I. Nilsson-Ehle, T.T. Hoshikawa, J.E. Edward, M.C. Schotz and L.B. Guze, J. Infect. Dis., 135 (1977) 414.
11 J.S. Wold, Antimicrob. Ag. Chemother., 11 (1977) 105.
12 J.P. Anhalt and S.D. Brown, Clin. Chem., 24 (1978) 1940.
13 I. Nilsson-Ehle, G. Kahlmeter and P. Nilsson-Ehle, J. Antimicrob. Chemother., 4 (1978) 169.
14 W. Mechlinski and C.P. Schaffner, J. Chromatogr., 99 (1974) 619.
15 A.J. Atkinson Jr. and J.E. Bennett, Antimicrob. Ag. Chemother., 13 (1978) 271.
16 T.B. Platt, J.D. Levin, J. Gentile and M.A. Leitz, in F. Kavanagh (Editor), Amphotericin B and Nystatin, Analytical Microbiology, Vol. 2, Academic Press, New York, 1972, pp. 147—185.