ELSEVIER

Journal of Chromatography B, 685 (1996) 135–140

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

New high-performance liquid chromatographic method for amphotericin B analysis using an internal standard

Maria Polikandritou Lambros*, S. Ali Abbas, David W.A. Bourne

The University of Oklahoma Health Science Center, College of Pharmacy, P.O. Box 26901, Oklahoma City, OK 73190, USA

Received 28 December 1995; revised 20 March 1996; accepted 25 March 1996

Abstract

A simple and reproducible HPLC method for the analysis of amphotericin B (AmB) in serum, lung and liver using natamycin as the internal standard was developed. AmB and natamycin were extracted from serum, lung and liver and were separated using an isocratic elution from a C_{18} reversed-phase column. The mobile phase consisted of acetonitrile–10 m*M* acetate buffer pH 4.0 (37:63, v/v). The HPLC system had two detectors in series. One was set at 303 nm and the other at 383 nm for the detection of natamycin and AmB, respectively. The retention times of AmB and natamycin were 15 and 6 min, respectively. The recovery efficiency was 96–70%. The limit of quantification was 0.1 μg/ml. The assay was reproducible, the within-day coefficient of variation ($n=6$) was <8% for serum, lungs and liver. The between-day variability ($n=6$) was <7.7% for serum, liver and lungs at 1 μg/ml or 1 μg/g tissue concentration. The assay was linear within the range 1–40 μg/ml ($r^2=0.999$).

Keywords: Amphotericin B

1. Introduction

Amphotericin B (AmB) is the most potent antifungal agent and the drug of choice in serious fungal infections. AmB binds to ergosterol, the sterol of fungal membranes and destroys them [1]. Its major limitation is its toxicity, mainly nephrotoxicity.

Encapsulation of AmB in liposomes reduces the toxicity and increases the therapeutic index of the drug [2,3]. Recently, several lipid AmB formulations for intravenous administration have been used in clinical trials [3,4]. We have developed several liposome AmB formulations and wish to study AmB kinetics following aerosolization. The purpose of this work is to develop and validate an HPLC method for AmB using an internal standard. This method should be capable of analyzing AmB concentrations not only in serum, but also, in lung and liver following an aerosol or intravenous administration.

Although several HPLC methods have been developed for AmB determination in biological fluids [5–12], the majority of them do not use an internal standard, and therefore samples have to be carefully controlled, otherwise errors can be introduced which can limit the reproducibility, precision and the accuracy of the method [13]. There are only a few methods for AmB analysis which utilize internal standards such as *N*-acetylamphotericin B [11], however this standard is not readily available. In

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00162-4

another method *p*-nitrophenol has been utilized as internal standard; the recovery of the method was 53–71% and the coefficient of variation was 18% from human plasma [14]. *p*-Nitrobenzyloxyamine has also been used as an internal standard in quantifying AmB. The recovery was about 75% and the reproducibility was 4%; however this method was only used to detect AmB in plasma or serum [10].

In this paper we describe the development of a new sensitive, accurate and reproducible method for analysis of AmB in serum, lung, and liver tissue.

2. Experimental

2.1. Chemicals

Amphotericin B was a gift from Bristol-Myers Squibb (Princeton, NJ, USA). Natamycin (pimaricin), methanol and acetonitrile were purchased from Sigma (St. Louis, MO, USA). Acetic acid was purchased from Mallinckrodt (Paris, KY, USA) and sodium acetate from Fisher Scientific (Fair Lawn, NJ, USA). All reagents were HPLC grade.

2.2. Chromatographic system and conditions

A Waters (Milford, MA, USA) high-performance chromatographic system which included a Waters 501 pump and a Waters 721 WISP autosampler was used. The system also included a tunable absorbance detector, Waters 486, which was used for the detection of AmB at a wavelength of 383 nm and another variable-wavelength detector, Waters 450, which was used for the detection of natamycin at λ=303 nm. The analytical column was μ-Bondapak RP-C_{18}, 10 μm, 3.9×300 mm. The guard column was an Alltech (Deerfield, IL, USA) direct connect refillable guard column, 2×30 mm packed bed filled with pellicular C_{18} refill. The mobile phase consisted of acetonitrile–acetate buffer 10 m*M* pH 4.0 (37:63, v/v). The injection volume was 100 μl. The flow-rate was 1 ml/min for the first 6 min and 2 ml/min thereafter.

2.3. Preparation of standard solutions

A stock solution was prepared by dissolving 10 mg of AmB in a mixture of methanol–DMSO (1:1, v/v). This solution was further diluted with a mixture of methanol–acetonitrile–1 m*M* acetate buffer pH 4.0 (5:4:1) to give a range of AmB concentrations of 0.1–40 μg/ml.

A 0.1-ml volume of natamycin suspension (2.5 g/100 ml) was dissolved in methanol and serially diluted to achieve 25 μg/ml. A 50-μl volume of natamycin solution along with 50 μl of each of the AmB concentrations were mixed with 0.5 ml of blank serum or 0.5 g of tissue and homogenized. The final amount of AmB per g of tissue or ml of serum was 0.1–40 μg. Nine replicates of each of the final concentration range samples were prepared and stored at −20°C for further analysis. Then each sample was mixed with 2 ml of methanol–acetic acid (9:1, v/v), vortex-mixed for 30 s and left in the dark for 1 h. The sample was centrifuged for 10 min at 1000 *g* and the supernatant was filtered through a MSI 0.45-μm filter (Westboro, MA, USA). A 100-μl aliquot of the filtrate was injected onto the HPLC system. The standard curve was constructed using the ratio of the area under the curve of AmB to the area under the curve of natamycin. The ratios of areas were plotted against the amount spiked. The amounts of AmB for the standard curve used was 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μg.

2.4. Tissue and serum extraction and preparation of the samples

A 50-μl volume of internal standard solution (25 μg/ml) were added to preweighed tissue (0.1–0.2 g) or serum sample (0.05–0.15 ml). The tissue samples were mixed with 0.5 ml of 1 m*M* phosphate buffer, pH 7.4 and homogenized using a manual glass homogenizer. The homogenate or the spiked serum samples were mixed with 2 ml methanol–acetic acid (9:1, v/v). The mixture was vortex-mixed for 30 s and left in dark for 1 h. Subsequently the sample was centrifuged for 10 min at 1000 *g* and supernatant was decanted and filtered (0.45 μm MSI, Westboro, MA, USA). A quantity of 100 μl of the filtrate was injected onto the HPLC system. During the analysis,

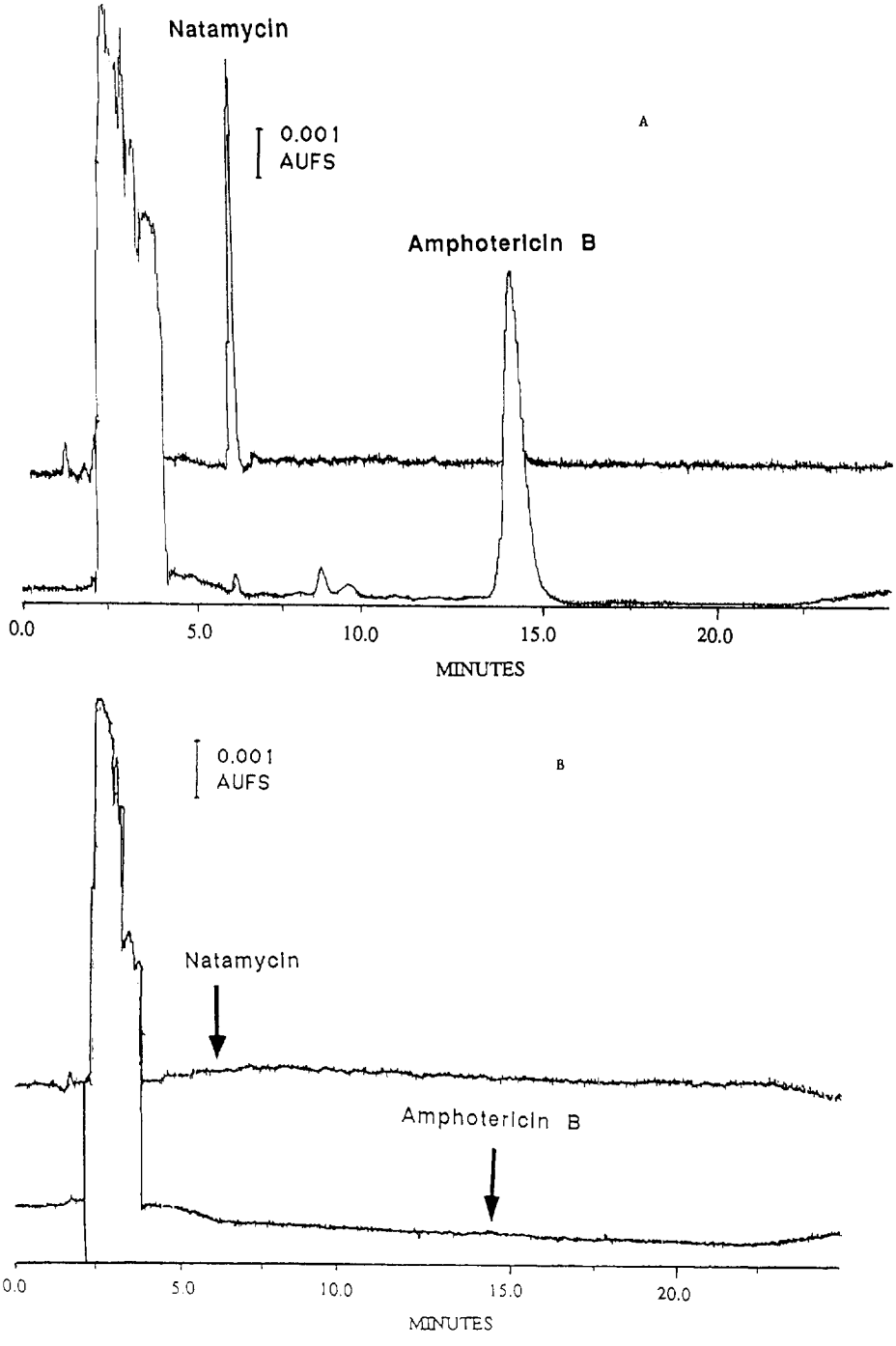

Fig. 1. (A) Typical chromatogram of AmB (5 μg) and natamycin (internal standard, 1.25 μg) in serum obtained using two detectors arranged in series. One detector was set at 383 nm for AmB and the other was set at 303 nm for the detection of natamycin. (B) Typical chromatogram of blank serum without natamycin and AmB.

care was taken to avoid exposure of the sample to light.

3. Results and discussion

Representative chromatographs of AmB and natamycin are shown in Fig. 1. The retention time for AmB was 15 min and the retention for natamycin was 6 min. The method exhibited linearity over the range 1–40 μg/ml (r^2=0.999) for serum, lung and liver. The column resolution was 11.2.

The lowest detectable concentration at a signal-to-noise ratio of 3:1 was 0.1 μg/ml. Better sensitivities have been reported in the literature, however, this method did not utilize an internal standard [12].

3.1. Recovery

The recovery study was performed by comparing the concentration in the serum, lung and liver spiked samples to the respective non-extracted standards (AmB in solution). The area under the peak of each serum, lung and liver sample was divided by the area under the curve of the quality control sample and multiplied by 100. The recovery for the internal standard was calculated by dividing the area under the curve of the natamycin in each sample by an average of areas under the curve of all non-extracted standards. The recovery results are shown in Table 1. Recovery efficiency for AmB in serum averaged 91.5%, in lungs 74%, and liver 70%. The average recovery for the internal standard was 83% in serum, 74% in lungs and 68% for liver. It has been reported that variations in precolumns influenced the recovery because of selective binding of AmB [14]. The recovery of the serum was 91.5% which is in agreement with some of the best AmB recoveries obtained in the literature [11].

Table 2
Within-day reproducibility

Sample (n=4)	Concentration spiked (μg/ml serum or μg/g tissue)	Concentration found (mean±S.D.) (μg/ml serum or μg/g tissue)	C.V. (%)	Accuracy (%)
Serum	0.25	0.26±0.016	6.70	4
	1.00	0.98±0.199	2.10	−2
	10.0	10.01±0.247	2.40	0.1
Lungs	0.25	0.28±0.017	8.02	12
	1.00	0.97±0.21	2.30	−3
	10.0	10.1±0.048	0.47	1
Liver	0.25	0.24±0.009	3.80	−4
	1.00	0.97±0.016	1.60	−3
	10.0	10.6±0.342	3.40	6

3.2. Variability and accuracy

The precision of the method was evaluated as variability within-day and between-day which were expressed as coefficient of variation (C.V.). Results from within-day and between-day variability are shown in Table 2 and Table 3. The within-day variabilities (n=4) of three AmB concentrations of serum, lung and liver ranged within 0.47–8.0%, while the between-day variability ranged within 0.8–13%.

The accuracy of the method was calculated as [(amount found−amount spiked)/amount spiked]×100. The results are summarized in Table 2 and

Table 1
Recoveries of amphotericin and natamycin internal standard

Sample	Recovery (%)		
(n=6)	Low concentration (0.1 μg/ml)	Medium concentration (1 μg/ml)	High concentration (5 μg/ml)
Amphotericin			
Serum	85.0	93.6	96.0
Lungs	72.7	75.0	74.2
Liver	67.0	71.0	71.0
Natamycin			
Serum	72.6	90.0	87.9
Lungs	80.1	71.3	72.8
Liver	64.0	70.0	69.0

Table 3
Between-day reproducibility

Sample	n	Concentration spiked (μg/ml serum or μg/g tissue)	Concentration found (mean±S.D.) (μg/ml serum or μg/g tissue)	C.V. (%)	Accuracy (%)
Serum	6	0.25	0.25±0.036	13.93	4.4
		1.00	1.03±0.062	5.99	3.4
		10.0	10.03±0.102	1.02	0.30
Lung	6	0.25	0.28±0.035	12.1	15.8
		1.00	1.02±0.022	2.2	1.9
		10.0	10.16±0.08	0.81	1.6
Liver	12	0.25	0.22±0.16	7.3	−10
		0.50	0.47±0.032	6.8	−4.3
		1.00	1.01±0.034	3.4	1.3

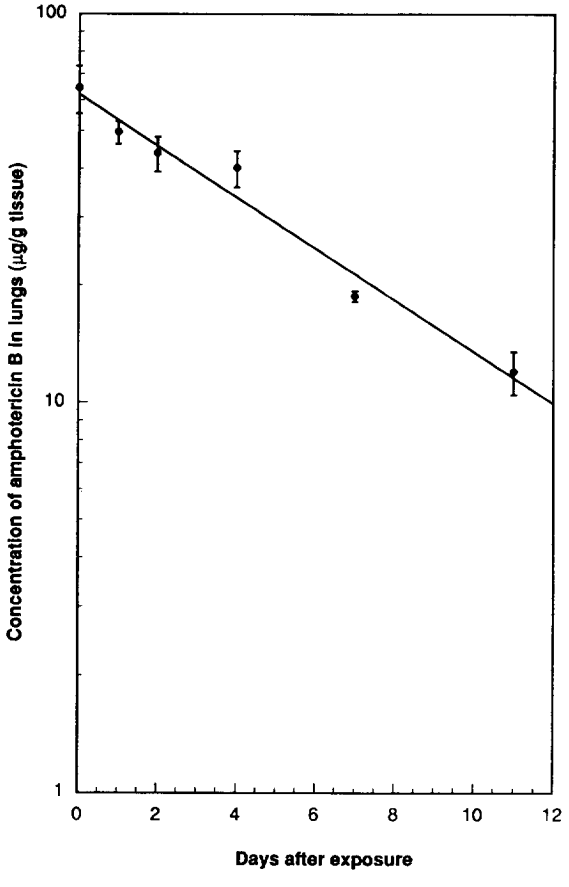

Fig. 2. Semilogarithmic plot of AmB concentration in lung versus time after administration of 0.7 mg/kg aerosolized AmB in negative liposomes. Symbols represent mean±S.D., $n=3$.

Table 3. The within-day accuracy was better than 12%. These data suggest that the method is accurate and precise.

3.3. Application of the method

The assay described above has been successfully applied to determine the AmB lung tissue concentration after administration of aerosolized liposomal AmB. Mice were administered aerosolized AmB encapsulated in negative liposomes and were killed at certain times after the dose. The mouse lungs liver and serum were analysed for AmB. Fig. 2 shows the lung concentration of AmB versus time. The concentration–time profile of the drug can be described by first order kinetics with a half-life of 4.5 days. As expected, AmB was not detected in serum and liver, at least within the sensitivity limits of the assay. Thus, aerosolization of liposomal amphotericin B may be a useful method of AmB administration for lung targeting which avoids the systemic toxicity of the drug.

4. Conclusion

The HPLC method described above is simple, fast, sensitive and reproducible. Moreover it uses a readily available internal standard, which further promotes its precision and accuracy. Blank rodent serum and tissues did not contain any endogenous materials that interfered with the chromatography. This meth-

od has been successfully used to study the pharmacokinetics of aerosolized liposomal AmB in mice.

References

[1] N.H. Georgopapadakou and T. Walsh, Science, 264 (1994) 371.
[2] J. Brajtburg, W.G. Powderly, G.S. Kobayashi and G. Madoff, Antimicrob. Agents Chemother., 34 (1990) 381.
[3] R.J. Hay, J. Infection, 28 (Supplement I) (1994) 35.
[4] V.J. Wiebe and M.W. Degregona, Rev. Infect. Dis., 10 (1988) 1097.
[5] C. Brassine, C. Laduron, A. Coune, J.P. Sculier, C. Hollaert, C. Meunier and F. Meunier, J. Chromatogr., 419 (1987) 401.
[6] P. Casaccia, A. Ladogana, Y.G. Xi, L. Ingrosso, M. Pocchiari, M.C. Silverstrini and A. Cittadinni, Antimicrob. Agents Chemother., 35 (1991) 1486.
[7] P.M. Kelly, J. Chromatogr., 437 (1988) 221.
[8] K.J. Christiansen, E.M. Bernard, J.W. Gold and D. Armstrong, J. Infect. Dis., 152 (1985) 1037.
[9] M. Margosis and A. Aszalos, J. Pharm. Sci., 73 (1984) 835.
[10] C.L. Golas, C.G. Prober, S.M. MacLeod and S.J. Soldin, J. Chromatogr., 278 (1983) 387.
[11] G.G. Granich, G.S. Kobayashi and D.J. Krogstad, Antimicrob. Agents Chemother., 29 (1986) 584.
[12] L.H. Wang, P.C. Smith, K.L. Anderson and R.M. Fielding, J. Chromatogr., 579 (1992) 259.
[13] S.W. Graves and S. Runyon, J. Chromatogr., 663 (1995) 255.
[14] J.W. Mayhew, C. Fiore, T. Murray and M. Barza, J. Chromatogr., 274 (1983) 271.