Journal of Chromatography, 278 (1983) 387—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1865

MEASUREMENT OF AMPHOTERICIN B IN SERUM OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received June 28th, 1983)

SUMMARY

A high-performance liquid chromatographic method for the analysis of amphotericin B in 25 μl of serum or plasma is described. The procedure involves the addition of the internal standard, *p*-nitrobenzyloxyamine, to the sample followed by a precipitation of protein with acetonitrile. The supernatant is directly injected into a chromatograph attached to a reversed-phase μBondapak (Waters) column containing C_{18} packing. The mobile phase is a 60 : 40 mixture of a sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile, and we employ a flow-rate of 1.5 ml/min and a detection wavelength of 405 nm. Total analysis time per sample is 10 min. Coefficients of variation were found to be less than 4% for concentrations less than 2 mg/l. Analytical recoveries were between 75 and 80%. No drug or drug metabolite interference was found. The method will be used to study pharmacokinetic and pharmacodynamic data in a pediatric population.

INTRODUCTION

Amphotericin is an antifungal compound which was isolated in 1956 from a soil actinomycete, *Streptomyces nodosus*, found in the Orinoco river area of Venezuela [1]. It exists in two forms, A and B; the latter, being more active, is used clinically. The basic moiety of amphotericin B (AMB) is aminodesoxyhexose, an aminomethyl pentose. It is active and useful clinically against a wide range of fungi including *Candida* species, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Sporotrichum schenckii*, *Aspergillus*, *Mucor*, and

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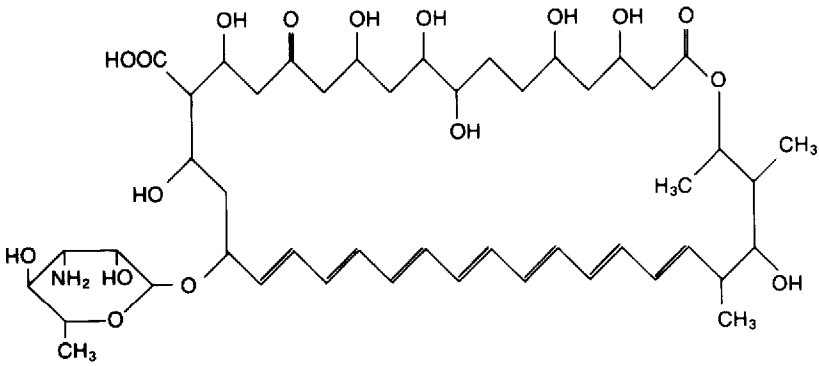
0378-4347/83/$03.00

Rhizopus. It is fungicidal against most of these fungi at concentrations of 1.0 mg/l or less [1].

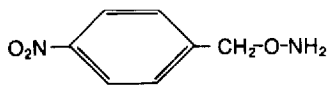
There is a limited amount of pharmacokinetic data on AMB, especially in children. The intravenous injection of 0.5—1.0 mg/kg yields peak plasma concentrations of 1.5—2.0 mg/l. There is no universal agreement on the method of intravenous administration. It is, however, common practice to treat fungal infections on the basis of a total dose of AMB. In general, a daily dose of 0.5—1.0 mg/kg will be administered for as many days as it takes to attain the desired total dose. AMB is perhaps the only antimicrobial agent which is frequently administered on the basis of a total cumulative dose rather than on the basis of a daily dose administered for an arbitrary period of time. Dosing on the basis of attaining plasma concentrations at least two- to three-fold in excess of the amount required to kill the specific fungi would seem to be a more rational approach. An accurate and reproducible analytical assay for AMB is necessary to permit this approach to dosing.

The most frequent and clinically significant side-effect of AMB therapy is nephrotoxicity [2]. Some degree of renal functional impairment occurs in almost all treated patients, and this is often a limiting factor to the total amount of drug which can be administered. Whether such renal dysfunction can be predicted and prevented by the monitoring of AMB serum concentrations, followed by appropriate dose adjustments, has not yet been established. Investigation of this possibility again requires the availability of an accurate and reproducible AMB assay.

The chemical properties of AMB make it a difficult drug to analyze. It has a high molecular weight (924.1), is poorly soluble in water and organic solvents,



Amphotericin B



p-Nitrobenzyloxyamine

Fig. 1. Structures of amphotericin B and the internal standard, *p*-nitrobenzyloxyamine.

and is sensitive to light, heat, air and pH below 6 or above 9 [3]. Only a few procedures exist for the measurement of AMB. These include colorimetry [4], microbiological assays [5], and high-performance liquid chromatography (HPLC) [4, 6]. The inherent problem with the colorimetric method is the absorption of hemoglobin at 405 nm. Lengthy analysis time and poor specificity and sensitivity are shortcomings with the bioassay. One HPLC method employs a sample volume of 1 ml, omits use of an internal standard, and requires many steps (deproteinization, centrifugation, filtration) prior to chromatography [4]. This paper [4] reports the superiority of the HPLC method over the bioassay procedure. A standard deviation of 3.3% (HPLC) compared to 32.6% (bioassay) was found. Another HPLC method incorporates an internal standard, but reports a fairly large coefficient of variation of 18% within a range of 0.08—10.0 mg/l [6].

We describe a rapid, accurate and sensitive micro procedure for the analysis of AMB in 25 μl of serum or plasma using *p*-nitrobenzyloxyamine (PNBA) as the internal standard. This method is capable of measuring AMB within the generally accepted, clinically significant range of 0.5—2.0 mg/l [1]. The chemical structure of this non-aromatic heptane antibiotic and that of PNBA are shown in Fig. 1.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Series 2/2 high-performance liquid chromatograph (Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 loop injector valve was used for the analysis. Chromatography was performed on a reversed-phase μBondapak 30 × 0.4 cm stainless-steel column, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.). The detector used was a Waters Model 441 spectrophotometer with 405-nm filter and adjusted to full-scale absorbance of 0.005 absorbance units. An Omniscribe B-5000 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used. Ultraviolet and visible scans of the drug and the internal standard were obtained using a PMQII (Carl Zeiss, Oberkochen, F.R.G.) spectrophotometer.

Reagents and standards

Acetonitrile and methanol were purchased from Anachemia (Mississauga, Canada). AMB was obtained from Sigma (St. Louis, MO, U.S.A.), while PNBA was purchased from Regis Chemical (Morton Grove, IL, U.S.A.). Dimethyl sulfoxide and sodium acetate were provided from Fisher Scientific (Fairlawn, NJ, U.S.A.). The mobile phase is sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile (60 : 40).

A stock standard solution of AMB (500 mg/l) was prepared in dimethyl sulfoxide while a working standard solution (2 mg/l) was prepared by dilution of the stock solution with the mobile phase. Control samples were prepared in drug-free sera to give concentrations of 0.5, 1.0 and 2.0 mg/l AMB. These solutions are stable for many months at −20°C. A 1 mg/l solution of PNBA was prepared in water and stored at 4°C. Solutions of PNBA could not be prepared in sodium acetate buffer or acetonitrile due to lack of solubility.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameter	Conditions
Column	μBondapak C_{18}
Mobile phase	Sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile (60 : 40)
Wavelength	405 nm
Flow-rate	1.5 ml/min
Temperature	Ambient temperature
Internal standard	*p*-Nitrobenzyloxyamine
Chromatography time	5 min
a.u.f.s.	0.005
Sample volume	25 μl

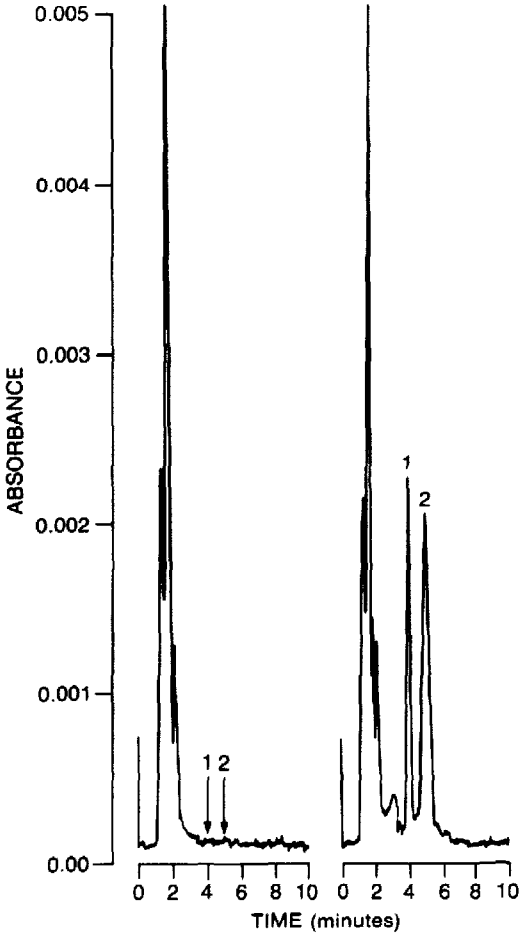


Fig. 2. Typical chromatograms of (left) a blank serum and (right) a serum sample containing 1 mg/l AMB. Peak 1 = internal standard, peak 2 = AMB.

Extraction procedure and conditions of analysis

Add 25 μl of internal standard (1 mg/l PNBA in water) to 25 μl of serum or plasma in a 6 × 50 mm glass test tube. Then acetonitrile (50 μl) is added to the solution which is vortexed for 15 sec. Centrifuge at 6500 *g* for 2 min. Inject 80 μl into the chromatograph. Table I lists the chromatographic conditions used in the analysis. Typical chromatograms for a serum sample and a serum blank are shown in Fig. 2.

RESULTS AND DISCUSSION

Analytical variables

Detector wavelength. Fig. 3 shows the absorbance scan for AMB and PNBA. AMB was found to have three absorbance maxima in the ultraviolet—visible spectrum. Initially a variable-wavelength spectrophotometer (Perkin-Elmer) was utilized for measurement of absorbance at the absorbance maximum of 384 nm. However, use of a fixed line source mercury lamp spectrophotometer and a wavelength of 405 nm provided better sensitivity, an improved signal-to-basesline noise ratio, and permitted use of a smaller sample volume. Both carbamazepine and phenytoin co-eluted with AMB and interfered with the measurement of AMB at 384 nm but not at 405 nm.

Column choice and column temperature. Studies using the 5- or 8-mm Radial Pak A columns (10 × 0.8 cm, Waters) resulted in poor band symmetry and provided undesirably broad peaks for AMB. Good peak symmetry with minimal band spreading were obtained with the μBondapak C_{18} columns. The retention time of AMB varied minimally with column temperature changes between 25° C and 40° C.

Standard and internal standard. No difference was noted between the pharmaceutical (Squibb Canada, Montreal, Canada) and chemical (Sigma) preparations of AMB. Both of these solutions contained a small peak with a retention time at approximately 3 min which probably corresponds to ampho-

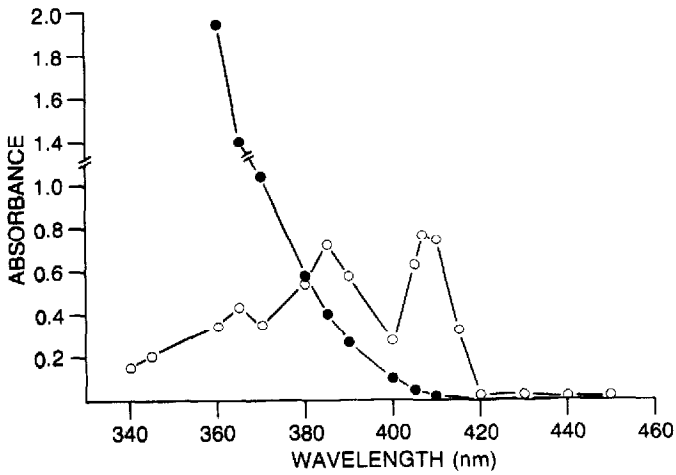


Fig. 3. Absorbance scan of 10 mg/l amphotericin B (○) and 2000 mg/l *p*-nitrobenzyloxyamine (●) in mobile phase.

tericin A. Amphotericin A is present as a trace contaminant in AMB preparations [7]. For purposes of quantitation of AMB, this contamination of the AMB standard was ignored.

Composition of the mobile phase. The optimal separation of AMB and PNBA was obtained when the buffer had a pH of 7.0 and a ratio of buffer to acetonitrile of 60 : 40. A buffer pH of 4.0, while having little effect on the retention time of the internal standard, significantly lengthened the retention time of AMB. Also, at this acidic pH amphotericin A coeluted with PNBA. Increasing the acetonitrile content shortened the retention time and improved the peak shape of AMB.

Method of calculation

The retention times of AMB and PNBA were determined by the daily injection of these compounds into the chromatograph. AMB was identified by its retention time relative to that of PNBA. A daily mean factor, based on the peak height ratios of AMB (A) to PNBA (B) observed in the three control sera, was derived. For example, in a control sample containing 2 mg/l AMB the calculation is as follows:

$$\frac{A}{B} \times \frac{1}{2} = \text{peak height ratio obtained for 1 mg/l AMB} = \text{factor.}$$

The average of the three resulting values produced the mean factor which was employed to calculate the controls and the unknown sample by the following formula:

$$\frac{\text{AMB peak height}}{\text{PNBA peak height}} \times \frac{1}{\text{mean factor}} = \text{AMB concentration in mg/l.}$$

Linearity

Injection of known amounts of AMB and PNBA demonstrated a linear relationship between peak height and concentration (Fig. 4). The serum concentration that corresponds to a full-scale recorder deflection is 3.9 mg/l AMB. This is an ample range for clinical interest. The sensitivity of the assay, i.e. peak height corresponding to twice the baseline noise, was found to be 0.1 mg/l.

Recovery

AMB was added to drug-free plasma to provide concentrations of 0.5, 1.0 and 2.0 mg/l. Recovery was determined by the replicate analysis (n = 10) of each of these three plasma pools. The peak heights obtained for AMB and PNBA were compared with the peak heights obtained by direct injection of working standard solutions. The results are summarized in Table II.

Precision

The between-day precision was assessed by analysing the three plasma pools for twenty working days. The coefficient of variation was equal or less than 4.0 at serum concentrations of 0.5—2.0 mg/l AMB (Table III).

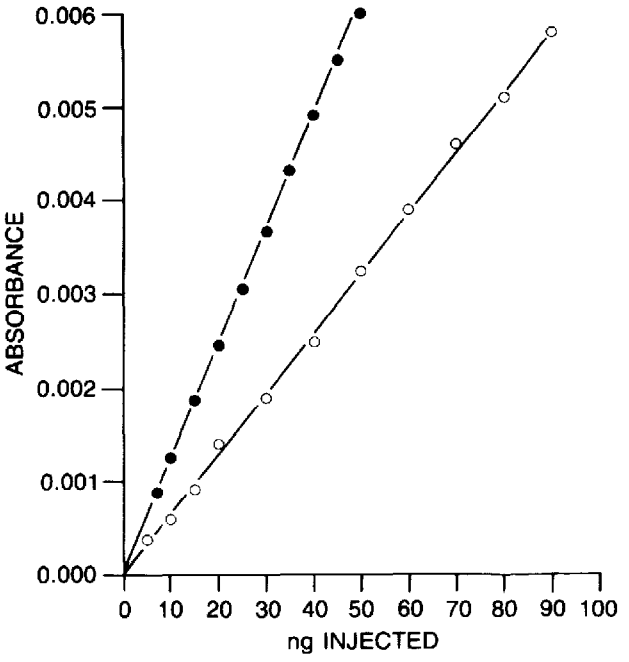


Fig. 4. Relative response curve for amphotericin B (○) and *p*-nitrobenzyloxyamine (●).

TABLE II

RECOVERY STUDIES

Compound	Concentration (mg/l)	Recovery (%) (n = 10)
AMB	0.5	80
	1.0	75
	2.0	78
PNBA	1000	95

TABLE III

BETWEEN-DAY PRECISION STUDIES

Compound	Concentration (mg/l)	C.V. (%) (n = 20)
AMB	0.5	3.4
	1.0	4.0
	2.0	3.1

Selectivity

Twenty sera obtained from pediatric patients known not to be receiving AMB were assayed without the presence of the internal standard. No interfering peaks were observed in the chromatograms. Further assessment of

TABLE IV

SPECIFICITY STUDIES

Antineoplastic	Antibiotic	Other
5-Fluorouracil	Gentamicin	Phenobarbital
Cytarabine	Ampicillin	Primidone
Cyclophosphamide	Cloxacillin	Carbamazepine
Methotrexate	Penicillin G	Ethosuximide
Vincristine	Trimethoprim	Phenytoin
Daunorubicin	Sulfamethoxazole	Theophylline
Allopurinol	Tobramycin	Folic acid
Thioguanine	Cephalothin	Diazepam
6-Mercaptopurine	Chloramphenicol	Furosemide
Cisplatin	Amikacin	Acetazolamide
Adriamycin		Metoclopramide
Dactinomycin		Acetaminophen
Flucytosine		Vidarabine
		Acetylsalicylic acid
		Dimenhydrinate
		Nabilone
		Chlorpromazine

TABLE V

STABILITY STUDIES

Days	Storage temperature		
	25° C	4° C	−20° C
	Concentration (mg/l)		
1	0.7	0.7	0.7
14	0.6	0.7	0.7
24	0.5	0.7	0.7

selectivity was determined by the direct injection of the aqueous solutions of the pure compounds listed in Table IV. None were found to interfere in the described method.

Stability studies

AMB was added to several drug-free plasma pools to give a concentration of 0.7 mg/l. Aliquots from these pools were stored in the dark at 25° C, 4° C and −20° C. Samples stored at each of these temperatures were analyzed on days 1, 14 and 24. The results are shown in Table V. As can be seen, AMB is stable when stored at either 4° C or −20° C for 24 days.

Future clinical application

We have described an accurate and reproducible procedure for the measurement of AMB. This will permit important pharmacokinetic and pharmacodynamic studies to be undertaken on this antifungal agent in the pediatric population. Based upon the monitoring of serum concentrations of AMB, we

hope to be able to rationalize our treatment schedules. We will also be able to investigate the possible relationship between nephrotoxicity and serum concentrations of AMB. If such a relationship exists, it may be possible to predict and hence prevent the occurrence of this side-effect.

ACKNOWLEDGEMENT

This work was supported in part by Research Grant PR922 from the Ontario Ministry of Health.

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