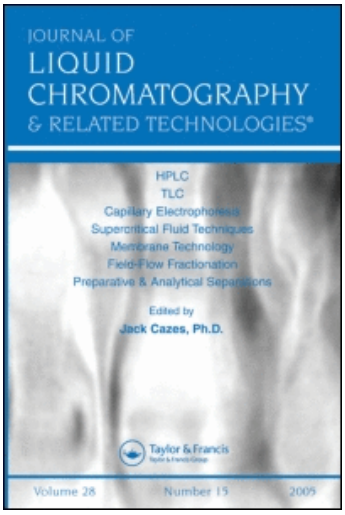

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DETERMINATION OF AMPHOTERICIN-B IN HUMAN PLASMA BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING A SHORT OCTYL COLUMN

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ABSTRACT

Amphotericin-B is a polyene antifungal antibiotic used for the treatment of severe systemic fungal infections. For effective treatment of urinary fungaria and the prevention of significant adverse-effects, monitoring the concentration of Amphotericin-B in biological samples of humans (ingesting the drug) is required. In this experiment, Amphotericin-B was isolated from plasma endogenous substances by adding 200 μL of acetonitrile in 800 μL of plasma. This mixture was vortex mixed, 20 mg of zinc sulfate and 10 mg of monobasic potassium phosphate was added to the mixture. This mixture was again vortex mixed and followed by centrifugation. The supernatant was filtered through a 0.45 μm membrane and a 100 μL aliquot of this solution was injected onto the chromatographic system. A short column of 60 mm x 4.6 mm packed with 3 μm octyl particles was used with an isocratic elution of 50/50, acetonitrile/0.01M KH_2PO_4 (v/v). The pH of the mobile phase mixture was adjusted to .3.5 with H_3PO_4. The intact

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drug molecule (parent drug) was monitored by a UV-visible detector at 410 nm and 0.10-0.005 A.U.F.S. The limits of detection of the method were 0.03 μg/mL for 100 μl injection volume at signal-to-noise ratio of 3.

INTRODUCTION

Amphotericin-B (AMB) is a heptaene antifungal antibiotic agent. In 1956, AMB was isolated from a soil actinomycete, Streptomyces Nodosus, in the Orinoco river area of Venezuela (1). Polyene macrolide antifungal agents are classified as tetraenes, pentaenes, hexaenes, and heptaenes according to the number of conjugated π-bonds in the molecule (2). AMB is a heptaene polyene which are found to have the strongest antifungal activity among all the polyenes.

AMB is extensively used for the treatment of most severe systemic fungal infections (3). An increased number of diabetic patients reported the incidence of urinary fungaria which can be treated with AMB. AMB is not found to be very effective in the treatment of urinary fungaria when administered orally; because 70-80% of the parent drug is not absorbed by the gut (4). Therefore, low blood or plasma concentration of the AMB and poor excretion in urine is observed frequently. The mechanism of AMB's antifungal activity is due to its antagonistic effect on ergosterol, which is present in fungal cells. The drug has no effect on cholesterol of normal cells (5).

AMB (trade name Fungizone) is usually administered intravenously with sodium desoxycholate because of its poor ability to be absorbed by the gut of the patient. The most severe

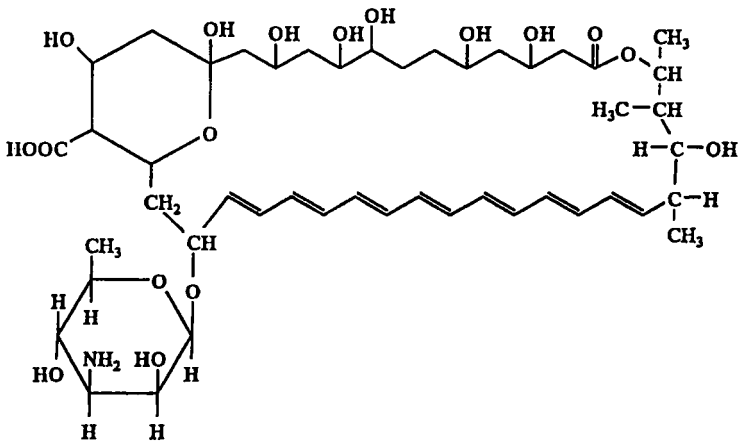

Figure 1. Chemical structure of Amphotericin-B (AMB)

adverse effect of AMB is nephrotoxicity which is significantly minimized when administered intravenously.

The procedures that are available for estimation of AMB in biological samples of patients include colorimetry, microbiological assays, and HPLC (1, 5-14). The colorimetry method has interference problem with the blood hemoglobin at 405 nm range. The microbiological assays takes longer, lacks selectivity, and has poor reproducibility. The first high performance liquid chromatography (HPLC) method to determine AMB in biological samples was published in 1977 (11). Several HPLC methods have been published recently (6-14). Most of these methods adapt multiple step sample preparation before chromatography. The reproducibility of some methods are also poor (at low concentration levels of AMB) because of poor sensitivity. Other

methods dilute the sample two to three times by adding organic solvents to precipitate proteins.

For practical purposes, a sensitive, rapid, and reproducible analytical method is required to monitor the concentrations of AMB in plasma or whole blood to obtain maximum therapy with minimum nephrotoxicity (by dosage optimization). The HPLC method described in this report can determine AMB in patients' plasma accurately and reproducibly at low concentration.

EXPERIMENTAL PROCEDURES

Apparatus

The HPLC system used was a Perkin-Elmer series 410B (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.), equipped with a Rheodyne 7275 sample injector with 100 μl sample loop (Rheodyne, Cotati, CA, U.S.A.). An Applied Biosystem Incorporated (ABI) UV-visible variable wavelength detector (Model 783) with a flow cell pathlength of 8.0 mm was used to monitor AMB (ABI Analytical, Ramsey, NJ, U.S.A.). The chromatograms obtained from the UV-visible detector was recorded on a Houston Instrument D5000 strip chart recorder (Houston Instrument Company, Austin, TX, U.S.A.). The analytical column used was a 60 mm x 4.6 mm, 3 μm octyl (slurry packed). The deionized water was obtained from a Milli-Q system (Millipore Corporation, Bedford, MA, U.S.A.). The centrifuge used for centrifugation of AMB plasma samples was purchased from Damon, Model HN (Damon, IEC Division, Needham Heights, MA U.S.A.). A Model 2200 Branson sonicator was used to

degas the eluent of the HPLC (Branson Cleaning Equipment Company, Shelton, CT, U.S.A.). A 5.0 mL gas tight syringe was used to filter the samples prior to injection into the HPLC (Hamilton Company, Reno, NV, U.S.A.). The 4.5 μm filter tip for the syringe was purchased from Rainin Instrument Company, Inc., (Woburn, MA, U.S.A.). Samples were vortex mixed on a vortex mixer obtained from Scientific Industries, Inc., (Bohemia, NY, U.S.A.). All quantitative volume transfer were conducted with a Gilson P-1000 digital pipette (Gilson International, Middleton, WI, U.S.A.).

Reagents, Solvents and Standards

AMB was obtained from E. R. Squibb & Sons (Princeton, NJ, U.S.A.). The aged pooled plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). The Econosphere 3 μm Octyl (C_8) particles were purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). HPLC grade acetonitrile was purchased from EM Science (Cherry Hill, NJ, U.S.A.). The phosphoric acid and monobasic potassium phosphate (KH_2PO_4) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Zinc sulfate was purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). The borosilicate glass culture tubes and disposable glass pipettes were purchased from Curtin Matheson Scientific (Elk Grove, IL, U.S.A.). All the solvents and reagents were used in the experiment as received without further purification or clean-up, except for the zinc sulfate and borosilicate culture tubes.

Slurry Packing of the Analytical Column

Slurry packing of the 60 mm x 4.6 mm, 3 μm, C_8 column was conducted by using a slurry packing procedure described elsewhere (15).

Cleaning of Zinc Sulfate and Borosilicate Culture Tubes

Cleaning of borosilicate disposable culture tubes and zinc sulfate were done according to a procedure described elsewhere (16).

Preparation of Stock Solution of AMB

A stock standard solution (0.20 mg/mL) of AMB was prepared in 80/20, MeOH/water (1% acetic acid) (v/v). Working standard solutions of AMB was prepared by diluting the above solution into appropriate concentrations. The original stock solution of AMB was stored frozen at -25°C and was found to be stable for at least 30 days. A standard solution of AMB (10 μg/mL) was injected into the HPLC system to determine the retention time under the experimental chromatographic conditions.

Isolation of AMB from Plasma Prior to Chromatography

Isolation of AMB from plasma prior to chromatography was done by adding 200 μL of neat acetonitrile in 800 μL of plasma. Approximately 20 mg of zinc sulfate and 10 mg monobasic potassium phosphate (KH_2PO_4) was added to the plasma-acetonitrile mixture. The pooled plasma was kept at -20°C until used. This frozen plasma was thawed at ambient temperature and 800 μL was transferred into a borosilicate culture tube. An aliquot of the standard stock solution of AMB was added to the 800 μL of plasma

and vortex mixed for 40 sec. A 200 μL aliquot of acetonitrile was added and vortex mixed for 30 sec. Approximately 20 mg of zinc sulfate and 10 mg of KH_2PO_4 was added to the above mixture (of plasma-acetonitrile-AMB) and vortex mixed for one min., and centrifuged at 2000 g for 5 min. The supernatant was decanted into another fresh borosilicate culture tube. This solution was filtered through a 0.45 μm membrane filter tip using a 5.0 mL gas tight syringe. 100 μL of this solution was injected into the chromatographic system.

Chromatographic Conditions

The mobile phase consisted of 50% acetonitrile in 0.01 M KH_2PO_4 dissolved in deionized water. The pH of the mixture was adjusted to 3.5 ± 0.1 with 6 M phosphoric acid. The flow rate of the mobile phase was 1.0 mL/min. The drug (AMB) was detected with a UV-visible absorbance HPLC detector at 410 nm and a sensitivity of 0.10 to 0.005 A.U.F.S. The concentration of AMB in patient's plasma sample was determined by comparing the peak height of the AMB in the patient's plasma sample with a standard calibration curve of AMB in human plasma.

Construction of the Calibration Curves of AMB in Human Plasma and Water

The calibration curve of AMB was constructed in two matrices; one in human plasma and another in water. At least six standard solutions of AMB were prepared in 20/80 (acetonitrile/plasma) and 20/80 (acetonitrile/water) by adding aliquots of the standard stock solution of AMB to give concentrations of about

0.10, 0.50, 2.0, 5.0, 10.0, and 20.0 μg/mL. The plasma and water solutions of AMB were treated as described in the section of "Isolation of AMB from Plasma Prior to Chromatography." A straight calibration curve of AMB peak height versus concentration was constructed and used for quantification of AMB in patient's plasma samples. The calibration curve was linear to at least 100 μg/mL. Therefore, if AMB concentrations in patient's plasma exceeds 20 μg/mL, then additional higher AMB concentration in plasma standard solutions are used to construct the calibration curve.

RESULTS AND DISCUSSION

Figure 2 is a typical chromatogram of pooled plasma containing no AMB which shows that no chromatographic peak from the plasma constituent elutes with the same retention time as that of AMB. Figure 3 is a typical chromatogram of a patient plasma (who has ingested the drug which shows that the AMB peak is baseline resolved from the peaks of plasma constituents.

AMB in patients' plasma samples were quantified by using a straight calibration curve of AMB peak height versus concentration instead of an internal standard method. Using a straight calibration curve method eliminated the possibility of any interference between the internal standard and the AMB peak, and also made the pre-chromatographic procedure of the method simpler to some extent. Two calibration curves were constructed, one in plasma and another in water. The standard solutions of AMB for

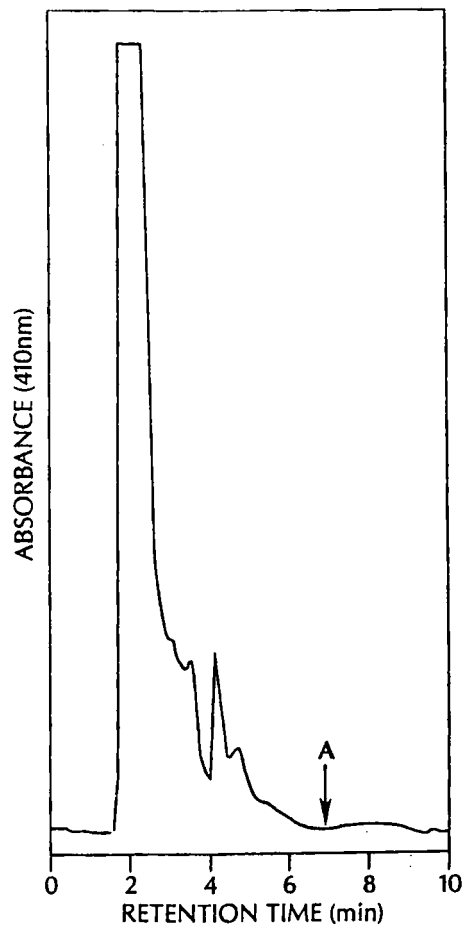

Figure 2. Typical chromatogram of pooled plasma sample containing no AMB. 'A' indicates the retention time of AMB. The detector was at 410 nm and 0.005 A.U.F.S.

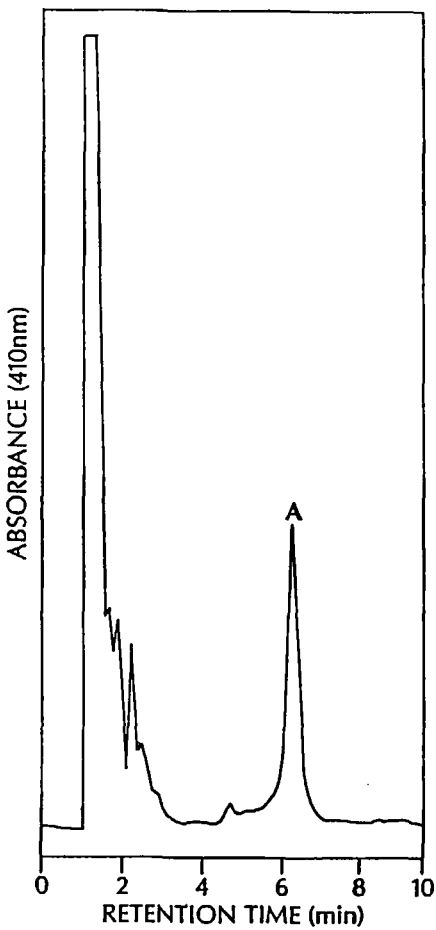

Figure 3. Typical chromatogram of a patient plasma sample who has ingested AMB. 'A' is the AMB peak. The detector was at 410 nm and 0.005 A.U.F.S. The AMB detected was
1.75 μg/mL.

both the calibration curves were treated identically. The objective of preparing these calibration curves was to investigate whether the acetonitrile, zinc sulfate and monobasic potassium phosphate, when used with water and plasma, had any effect on the slope and other parameters of the two calibration curves. The slope and y-intercept of the calibration curve of AMB in water was 18.84 and -3.24, and in plasma the slope and y-intercept was 14.70 and -0.19. The ratio of the slopes of the curves in plasma and water indicate that about 22% of the drug may have coprecipitated with plasma endogenous substances during pre-chromatography sample preparation. The y-intercepts of the two curves are also different. Therefore, one must use a calibration curve of AMB in plasma for determination of the drug concentration in patient's plasma samples. The 20% acetonitrile used in the isolation of AMB from plasma precipitated significant amounts of protein (probably of high molecular weight). The addition of zinc sulfate caused more precipitation of plasma proteins which were not affected by acetonitrile. The addition of monobasic potassium phosphate in sample preparation was necessary to obtain good recoveries of AMB.

The cleaning of zinc sulfate and borosilicate culture test tubes is very critical for this method to achieve reproducible chromatograms with no interfering peaks from the blank. Large interfering peaks were observed (occasionally) if cleaning of zinc sulfate and culture test tubes were not conducted prior to sample preparation and pre-chromatographic isolation of AMB from plasma.

A guard column was installed between the injector and the analytical column and was used throughout the study. The guard column was packed with the same stationary phase as that of the analytical column. the analytical column showed stable chromatographic properties up to at least 200 injections of plasma samples. The stationary phase of the guard column was replaced with fresh packing after injection of 30 to 40 plasma samples because of the back pressure of the chromatographic system increased up to 5,000 p.s.i.

Extraction of AMB from aqueous sample with organic solvents was investigated using acetonitrile, methanol, isopropanol, and n-propanol. The organic solvents were salted-out from the aqueous mixture by saturating the solution with ammonium sulfate or with anhydrous potassium carbonate. The extraction efficiency of AMB for all the organic solvents were poor and nonspecific for the drug. Table 1 shows the data obtained from this experiment. Inspection of the data reveals that acetonitrile extracted the drug with minimum efficiency and n-propanol showed the highest extraction efficiency. All of these solvents also extracted plasma endogenous materials which eluted with similar retention times as that of AMB. Therefore, extraction of AMB with organic solvents was eliminated in this experiment.

The reproducibility of the method described in this report was determined by multiple analyses of plasma samples fortified with aliquots of standard AMB solution with concentrations between 0.2 μg/mL and 10.0 μg/mL. Table 2 shows the assay precision for

Table 1

Extraction Efficiency of AMB from Aqueous Standard Solutions

Concentration	Percentage extracted (Mean ± S.D., n=5)			
(μg/mL)	Acetonitrile[a]	Methanol[b]	Isopropanol[a]	n-Propanol[a]
5.0	28 ± 7	51 ± 6	42 ± 6	58 ± 7
10.0	30 ± 4	49 ± 3	41 ± 3	56 ± 4
20.0	27 ± 3	52 ± 4	43 ± 4	59 ± 3
50.0	28 ± 2	54 ± 3	43 ± 2	55 ± 4

[a]Ammonium sulfate was used to salt-out the solvents
[b]Anhydrous potassium carbonate was used to salt-out methanol

Table 2

Precision and Accuracy Data for Within-Day Analysis

Actual Concentration (ng/mL)	Concentration Determined[a] (ng/mL) Mean ± S.D. (n=5)	Relative Standard Deviation (%)	*Accuracy (%)
200	800 ± 13	7.2	90
500	520 ± 19	3.6	104
2000	1900 ± 97	5.1	95
5000	5300 ± 110	2.0	106
10000	10500 ± 340	3.2	105

$$* \text{ Accuracy} = \frac{\text{Concentration determined}}{\text{Actual Concentration}} \times 100$$

Table 3

Precision and Accuracy Data for Day-to-Day Analysis

Actual Concentration (ng/mL)	Concentration Determined[a] (ng/mL) Mean ± S.D. (n=5)	Relative Standard Deviation (%)	*Accuracy (%)
200	220 ± 14	6.4	110
500	480 ± 25	5.2	96
2000	2150 ± 58	2.7	108
5000	4850 ± 170	3.5	97
10000	9900 ± 280	2.8	99

*Samples were assayed every other day for five days

within-day analyses and Table 3 shows the precision for day-to-day analyses. Reviewing the data of Table 2 and Table 3 indicates that the reproducibility (percent of relative standard deviation) for within-day and day-to-day analyses is better at higher concentrations of AMB. This may be due to higher uncertainty of the analyte signal (at low concentration of AMB) because of the noise of the UV detector at higher sensitivity.

The recovery of AMB from human plasma was determined by adding an aliquot of the drug standard solution to the plasma samples to give twice the concentration of AMB that had been determined earlier. After fortifying with standard AMB solution, the plasma samples were treated identically as before. The concentration was determined by injecting an aliquot of the sample

Table 4

Recovery of AMB in Patients' Plasma Samples

Mean Concentration (μg/mL) (n=3)			
In Plasma	Expected After Addition	Determined After Addition	Percent Recovery
0.45	0.90	0.96	106.6
1.30	2.60	2.50	96.2
0.85	1.70	1.60	94.1
1.75	3.50	3.65	104.3

into the HPLC system. The recovery of AMB from plasma varied from 94.1% to 106.6% over a concentration range of 0.45 μg/mL to 1.75 μg/mL. The summarized data of the recovery experiment has been tabulated in Table 4.

It is difficult to obtain sharp and symmetric chromatographic peak for AMB using typical HPLC columns. A broad peak of AMB is usually obtained. The broad peak may be due to poor mass-transfer of the AMB molecule in mobile phase and also on the stationary phase. Different columns were investigated for AMB to obtain the one with maximum efficiency and selectivity. The columns investigated are listed in Table 5.

The results of the above experiment indicates that the polymeric base columns (PRP-1) showed very low column efficiencies and sensitivity. The column efficiencies for ODS and octyl

Table 5

Investigation of HPLC Columns Packed with Different Stationary Phases for AMB Analysis

Column	Theoretical Plates (Plate/m)	Mean Peak Height (cm) (n=3)
6 cm x 4.6 mm PRP-1, 10 μm	4,500	16.8
15 cm x 4.1 mm PRP-1, 10 μm	3,600	12.3
10 cm x 4.6 mm 3 μm, ODS	7,200	14.7
10 cm x 4.6 mm 5 μm, Octyl	6,400	15.2
6 cm x 4.6 mm 3 μm, Octyl	8,300	23.9

Flow rate = 0.8 mL/min.
AMB injected = 0.90 μg for all the columns

stationary phases were comparable. The 6cm octyl (C_8) column with 3 μm particle size was used in this experiment because of highest sensitivity due to the sharp chromatographic peak. The chromatographic band broadening due to longitudinal and eddy diffusion was less for the column used in the final method than for the other columns due to small particle size of the stationary phase (3 μm) and short length (60 mm) of the column. Using the short column also decreased the chromatographic time of AMB significantly. The efficiency of the analytical column deteriorated occasionally, resulting into broad AMB peaks. This

problem was eliminated by washing the column overnight with 1.0% trifluoroacetic acid in 50/50, MeOH/CH_3CN solution.

The standard procedure for the sample preparation described in the literature (12-14), uses one to three milliliters of organic solvent. In this experiment, the sample is diluted only 20% by adding 200 μL of acetonitrile in 800 μL of plasma. Therefore, the sensitivity of the method improved over the previous methods. Also, other methods inject the sample directly after precipitation of the protein with an organic solvent. Small particles and colloidal materials in the protein- precipitated samples deteriorate the performance of the analytical column significantly. Addition of a filtering step in the sample preparation procedure of this experiment prolonged the performance of the analytical column.

The HPLC method presented in this report can be used to conduct the in-vivo pharmacokinetic studies of AMB in humans. Interference of some common prescription and non-prescription drugs were tested; famotidine, cimetidine, ranitidine, creatinine, and caffeine all showed negative results.

CONCLUSION

The analytical method presented in this paper to determine AMB from human plasma is simple, rapid and sensitive. AMB assays described by other authors in the literature are relatively complex or time-consuming or both. The sensitivity of many methods are not adequate to conduct in-vivo pharmacokinetic study in

humans who has ingested the drug. The extraction of the drug and evaporation of the extracting solvent prior to chromatography have been eliminated from the procedure, which increased the reproducibility of the method and reduced the time for sample preparation. Loss of AMB during filteration through 0.45 mm membrane was investigated and was found to be negative. The limit of detection (LOD), using a detection wavelength of 410 nm was 0.03 μg/mL for a 100 μL sample volume. This method may be used to conduct in-vivo pharmacokinetic, bioavailability and toxicokinetic studies.

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