Determination of amphotericin B in human plasma using solid-phase extraction and high-performance liquid chromatography

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Abstract

A rapid and selective HPLC method is described and validated for measuring amphotericin B (AB) in plasma. The procedure involves the solid phase extraction of AB from plasma by incorporating 1-amino-4-nitronaphthalene as an internal standard during the last elution step in extraction followed by HPLC analysis with UV detection at 407 nm. The chromatographic separation is achieved in less than 10 min on a reversed-phase C-18 column using acetonitrile-disodium edetate (20 mM) (45:55, v/v) at pH 5.0 as eluent. A linear response over the concentration range of 0.0100–2.00 µg ml^{-1} is obtained having a detection limit of 0.00500 µg ml^{-1} for AB. The mean extraction recovery is found to be $98.1 \pm 1.1\%$ ($n = 15$). The within-day and day-to-day R.S.D. were less than 2% ($n = 15$) and 6.54% ($n = 45$) respectively. This method is applied for quantifying AB trough levels in the plasma of cancer patients who have been on antifungal therapy with AmBisome®. It can further be applied either for AB therapeutic monitoring or single/multiple pharmacokinetic analysis of AB in plasma. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

The polyene antibiotic Amphotericin B (AB), a natural fermentation product of *Streptomyces nodusus*, is a member of macrocylic compounds containing both carboxyl and amino groups. AB has an amphipathic character due to the rigid lipophilic chain of seven conjugated double bonds on one side of the macrolide ring and the hydroxyl groups on the opposite side and this characteristic structure is believed to be important in its biological action [1]. Despite a broad sectrum of antifungal activity, administration of AB as a mixed-micellar dispersion with sodium deoxycholate (Fungizone®, Bristol-Myers Squibb, USA) is limited by severe dose-dependent toxicity. To

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reduce this toxicity several lipid-based formulations of AB have been developed, such as a liposomal formulation (AmBisome®, NeXtar Pharmaceuticals, USA), a lipid complex of AB with phospholipids (ABELCET®, Liposome Company Inc., USA) and a colloidal dispersion of AB with cholestryl sulfate (Amphocil®, Sequus Pharmaceuticals, Inc., USA), and they are now available in several countries [2–5]. Although each of these novel products has its own pharmaceutical composition and pharmacokinetics, there has been still limited data about their optimal therapeutic dosages and disposition kinetics [2,4,6–13]. In this respect, there has been a current interest and need for rapid and reliable measurements of AB plasma, whole blood and tissue concentrations by using validated assays [2,14]. Several HPLC methods aiming at quantifying AB in biological fluids and tissues have been reported after applying either liquid–liquid extraction or solid-phase extraction (SPE), and most of them were based on the procedures described by Nilsson-Ehle et al. [15], Warnock et al. [16], Golas et al. [17], Mayhew et al. [18], Bach [19], Granich et al. [20], Brasinne et al. [21], Wang et al. [22] and Wasan et al. [23] with or without modifications [4,6,9,24–29]. Among these procedures, the sample pretreatment using the SPE has been shown to offer the required specificity by preventing the endogenous peaks caused by the biological matrix and *N*-acetyl amphotericin B has been one of the recommended internal standards especially for the SPE procedures [20]. However, this substance is not commercially available and due to the difficulties in its supply, the sample pretreatment involving the SPE of AB were achieved without using it or any other internal standard as described in several studies [4,30–32]. The other internal standards, such as *p*-nitrobenzyloxyamine [17], *p*-nitrophenol [18], yellow 42 dye [19], 1-amino-4-nitronaphthalene [3,20,24,27] and natamycin [33], were incorporated during the liquid–liquid extraction procedures due to their solubility and spectrophotometric properties. However, it is well known that the use of an internal standard in an HPLC method involving either SPE or liquid–liquid extraction compensates for several technical variations occurring not

only during extraction and evaporation, but during the chromatographic manipulations as well [3,20]. Its use further was shown to improve the performance characteristics of analytical parameters examined in the assay validation [14].

Consequently, the purpose of the present study was to establish a rapid and reliable HPLC method for quantitation of AB in human plasma that could be performed easily in research and routine clinical laboratories with currently available equipment and materials. In this respect, a modified HPLC method was established by evaluating previously published HPLC assays for AB quantitation from plasma and serum [15–32]. A cheap and readily available internal standard, such as 1-amino-4-nitronaphthalene, was used during the SPE of AB from plasma, but it was incorporated into the last elution mixture and co-eluted with AB in order to control the extraction procedures and also the HPLC runs. Since validated bioanalytical methods should be used during in vivo drug analysis and pharmacokinetic investigations, a validation protocol was established by taking the recommendations of the conference on 'Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies' [34], the guideline announced by the International Conference on Harmonization (ICH) on 'Validation of Analytical Procedures: Methodology' [35] and some others into consideration [36,37].

2. Experimental

2.1. Materials

HPLC grade acetonitrile and methanol (MeOH) (E., Merck, Darmstadt, Germany) were used during the analysis. AB (Bristol-Myers Squibb, Princeton, NJ, USA) was obtained from Er-Kim Drug Inc., (Istanbul, Turkey). 1-Amino-4-nitronaphthalene as the internal standard was supplied from Aldrich (Steinheim, Germany). Analytical grade dimethylsulfoxide (DMSO), disodum edetate, disodium hydrogenphosphate, potassium dihydrogenphosphate, and sodium chloride were purchased from E., Merck, (Darmstadt, Germany). Deionized water was obtained

by using a Millipore Milli-Q purification system (Millipore, Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

Analysis was performed using a Shimadzu (Kyoto, Japan) HPLC system consisting of a Model LC-6A pump, a Model SPD-6AV UV–visible spectrophotometric detector and a Chromatopac CR3A integrator. The system was operated at ambient temperature at a flow-rate of 1.0 ml min^{-1} using a Bondapak C-18 μm reversed-phase column (300 × 4.6 mm i.d., dp = 5 μ) (Waters, Milford, MA, USA). Elution was carried out isocratically with acetonitrile–disodium edetate (20 mM) (45:55, v/v) at pH 5.0. The mobile phase was filtered through a 0.45 μm nucleopore membrane filter (Costar, Bodenheim, Germany) and degassed using a sonication bath for at least 1/2 h. A Rheodyne Model 7125 injection valve (Rheodyne Inc., Cotati, USA) with a 100 μl fixed sample loop was used for sample injection. The UV detection wavelength was 407 nm based on the UV spectrum of AB in the mobile phase, which was recorded by a UV–visible 16 Spectrophotometer (Shimadzu, Kyoto, Japan) with 0.1 AUFS.

2.3. Solid-phase extraction

For the SPE, Sep-Pak Cartridges of 1 ml capacity (C-18, 100 mg, Waters, Milipore, Milford, MA, USA) and PH-EC columns of 1 ml capacity (Phenyl, 100 mg, Internationale Chromatographie Technologie (ICT), Basel, Switzerland) were used. The Vac-Elut vacuum chamber was obtained from Varian (Harbor City, CA, USA). The SPE columns were attached to the Vac-Elut chamber and conditioned by passing 2 ml of MeOH, 2 ml of water and 3 ml of phosphate buffer (pH 7.4; 11 mM) that was prepared according to the British Pharmacopoeia [38] at flow rates of about 1 ml min^{-1}. One ml of the sample, such as the plasma sample, the standard working solution, the quality control sample or the sample for stability study, was used during the SPE. The sample to be extracted was loaded to the SPE column at a rate of about 0.2 ml min^{-1} and washed with 3 ml of MeOH–phosphate buffer (pH 7.4; 11 mM)

(40:60, v/v) three times with a flow rate of about 1 ml min^{-1}. After vacuum application for 2–3 min, 1 ml of acetonitrile–disodium edetate (2.5 mM) (60:40, v/v) at pH 4.2 containing 1 μg ml^{-1} of 1-amino-4-nitronaphthalene was added. AB and the internal standard were eluted together and finally 100 μl of the extract was injected directly into the analytical column.

2.4. Preparation of plasma samples

The venous blood samples were obtained from healthy human volunteers and from two cancer patients on antifungal therapy with AmBisome®. Prior the blood sampling, informed consent was obtained from the patients according to the protocol approved by the Institutional Review Board of the Hacettepe University [14]. All of the blood samples were collected into the vacutainer tubes containing tripotassium edetate as an anticoagulant and centrifuged immediately for 10 min at 1500 × g. During the validation of the assay and construction of calibration curves fresh human plasma was used. The plasma obtained from the patients were divided into two fractions and kept frozen at −20°C and duplicate measurements were done for each fraction. Before SPE, each fraction of the patient plasma was allowed to thaw at room temperature in the dark and without applying heat or ultrasonication. After adding 1 ml of phosphate buffer (pH 7.4; 11 mM) to 1 ml of the plasma, the SPE was performed with 1 ml of the diluted plasma.

2.5. Standard solutions

The stock standard solutions of AB (500 μg ml^{-1}) and 1-amino-4-nitronaphthalene (500 μg ml^{-1}) were prepared by dissolving 5.00 mg of each of them separately in DMSO–MeOH (1:1, v/v) mixture. The working standard solutions for constructing calibration curves and for assay validation covering the range for intended use (1–200%) were obtained from blank plasma spiked with various amounts of the appropriately diluted stock solution of AB. In this respect, first the stock solution of AB was diluted with deionized water to the concentrations of 50.0, 10.0 and 1.00

μg ml^{-1} of AB and the required aliquots taken from these solutions were spiked into the plasma. After adjusting the volume of each working standard solution to 1 ml with the plasma, 1 ml of phosphate buffer (pH 7.4; 11 mM) was added and vortexed. Finally, 1 ml of the resulting solution was used for the SPE.

2.6. Quality control samples

On every working day, the quality control plasma samples in triplicates at three different concentrations, one near to the lower range (0.0150 μg ml^{-1}), one in the mid range (1.00 μg ml^{-1}) and one in the upper range (1.80 μg ml^{-1}) of the calibration curve (1.50–180%) were prepared and extracted in the manner described in the SPE. They were incorporated randomly into each run during the construction of calibration curves and along with the measurement of unknown samples. The individual and overall mean percent recovery of AB from the quality control samples were calculated and R.S.D. values of maximum 15% provided the basis of acceptance criteria for the run.

2.7. Sample preparation for AB stability in plasma

For the stability study the standard working solution in plasma at 1.00 μg ml^{-1} of AB was used and prepared as described previously. The samples were stored in hermetically closed vials under the following conditions: at 25°C exposed to fluorescent light and at 25, 4 and −20°C in the dark. One ml of the sample withdrawn from each vial at the beginning and at the end of 1st, 10th, 30th days was extracted as described in the SPE section. Each sample was assayed in triplicate by HPLC under the final assay conditions and the mean percent of drug remained starting from the time zero were calculated

2.8. Preparation and storage conditions

All procedures during the preparation and the SPE of the plasma samples, the standard solutions and the quality control samples till chro-

matographic analysis were completed in a dark room. The stock solutions, the standard working solutions and the plasma samples were kept at −20°C wrapped in aluminum foil in order to prevent AB degradation

2.9. Validation of the assay

2.9.1. Linearity

The linearity was evaluated with seven blank plasma spiked with various amounts of appropriately diluted stock standard solutions to form working solutions of plasma containing 0.0100, 0.0500, 0.100, 0.500, 1.00, 1.50 and 2.00 μg ml^{-1} of AB. For each concentration three measurements were performed and calibration curves based on the peak height ratio (PHR) of AB to the internal standard versus nominal concentrations expressed in μg ml^{-1} of AB were constructed. The data were statistically evaluated by using a regression analysis software package called EKKAR4.1 and the equations were calculated by least-square linear regression analysis.

2.9.2. Accuracy and recovery

In the accuracy evaluation three working standard solutions in plasma covering the calibration range and containing 0.0100, 1.00 and 2.00 μg ml^{-1} of AB were prepared as described previously. Five consecutive measurements were performed for each concentration within the same day. The SPE recovery was calculated by comparing the PHR of AB to the internal standard obtained from the extracted working standard solutions in plasma and those resulting from the direct injection of the working standard solutions of AB prepared in the mobile phase having the same concentrations of AB and the internal standard. The individual and the overall mean percent recovery were calculated and a R.S.D. value of maximum 15% was set as the acceptance criteria.

2.9.3. Precision

The within-day and day-to-day analytical precision were determined by analyzing three differ-

ent working standard solutions in plasma having 0.0200, 1.00 and 1.75 µg ml^{-1} of AB. Five consecutive measurements were performed for each concentration within the same day and on three different days. The acceptance criteria for within-day and day-to-day R.S.D. values were not more than 15%.

2.9.4. Sensitivity

The sensitivity in terms of detection and quantitation limits were determined by using two different working standard solutions in plasma having 0.00900 and 0.0100 µg ml^{-1} of AB, which were prepared as described in the standard solutions and the SPE sections. The concentration measured with suitable accuracy and precision was accepted as the limit of quantitation. The limit of detection at a signal-to-noise ratio of about 3:1 was regarded as the lowest detectable amount.

2.9.5. Selectivity

The selectivity of the HPLC method was checked by analyzing different independent blank human plasma samples. The chromatograms of the blank plasma samples were compared with the chromatograms obtained by plasma spiked with AB and the plasma spiked with ten different drugs that were potentially used during the therapy of cancer patients who had fungal infections. Furthermore, the chromatograms obtained from the plasma samples of the cancer patients who were on antifungal therapy were examined for possible interference.

3. Results and discussion

The optimum conditions for the SPE of AB from the plasma were established after performing preliminary experiments with phenyl (PH-EC) and octadecyl (Sep-Pak) columns [14]. The SPE procedure involving phenyl columns described by Bach et al. [19] produced inconsistent results and low AB recovery in our experimental conditions (data not shown). A rapid and an efficient SPE of AB from the plasma was achieved by using C-18 columns according to Granich et al. [20] and Wang et al. [31] with modifications. The SPE procedure reported by Wang et al. [31] did not involve any internal standard application. On the contrary to the study presented by Granich et al. [20], in which *N*-acetyl amphotericin B was the internal standard during the SPE of AB from serum, in our study 1-amino-4-nitronaphthalene was used as the internal standard. It was incorporated during the last elution step of the extraction procedure, while its high solubility in organic solvents prevented its addition during the first step. Since the pH of the final elution solvent or the buffer used during SPE by Wang et al. [31] was not reported, in our procedure the pH value was optimized and a successful extraction efficiency for AB was observed with the final elution solvent containing acetonitrile – disodium edetate (2.5 mM) (60:40, v/v) at pH 4.2. Finally, the results dealing with the extraction efficiency in our procedure, which covered the calibration range of 0.0100–2.00 µg ml^{-1} of AB were satisfactory and better than previously reported procedures [20,31,33]. Data on the accuracy evaluation of our

Table 1
Accuracy of the method for analysis of AB in spiked plasma samples

Concentration (µg ml^{-1})	Added (%)	Found[a] (%)	Recovery[b] (%)	R.S.D. (%)
0.0100	1	0.938 ± 0.024	93.9 ± 2.4	5.81
1.00	100	99.9 ± 0.3	99.9 ± 0.3	0.762
2.00	200	202 ± 2	100 ± 1	1.80
Mean ± S.D., $n = 15$			98.1 ± 1.1	4.51
95% confidence limits			$101 > \mu > 95.8$	

[a] Calculated from peak height ratio (AB/IS) and expressed as found amount in percent, mean ± S.D., $n = 5$.
[b] Calculated from peak height ratio (AB/IS) and expressed as recovery in percent, mean ± S.D., $n = 5$.

method and the analytical recovery of AB determined was illustrated in Table 1. The individual SPE recovery at low, medium and high concentrations were $93.9 \pm 2.4\%$ (R.S.D. 5.81%), $99.9 \pm 0.3\%$ (R.S.D. 0.762%) and $100 \pm 1\%$ (R.S.D. 1.80%) respectively, whereas the mean SPE recovery at all concentrations was $98.1 \pm 1.1\%$ (R.S.D. of 4.51%, $n = 15$). In the study of Wang et al. [31], although the calibration range was between 0.005 and 2.50 µg ml^{-1} of AB, the extraction efficiency reported as an average (92%) covered the range of 0.025–2.50 µg ml^{-1}. As a result, not only the extraction efficiency at the lowest concentration (0.005 µg ml^{-1}), but the other validation parameters, such as accuracy and precision over the calibration range defined, was not taken into consideration in their study. In this respect in our study an improvement in the recovery has been shown and this achievement was due to the combination of carefully elaborated procedure of the SPE and the sample handling during preparation and storage conditions, since AB was also found to be light sensitive. In addition to that, the use of the internal standard controlled the variations, especially during the chromatographic runs and resulted in reproducible extraction recoveries and thus improved the performance of the validation.

Reproducible chromatographic separation of AB and the internal standard were established after examining various chromatographic conditions by injecting 100 µl of the extracted plasma sample without concentrating. The resolution of the internal standard was not achieved when MeOH was present as a third component in the mobile phase mixture of acetonitrile and disodium edetate as reported by Kan et al. and Bach [10,19]. For this reason, binary mixtures of acetonitrile and disodium edetate in different compositions were examined. However, poor chromatographic resolution was observed by using the mobile phase consisting of acetonitrile – disodium edetate (10 mM) (40:60, v/v) at pH 4.2 according to Granich et al. [20]. In their study, the chromatographic separation of AB and 1-amino-4-nitronaphthalene in serum was achieved after applying a liquid–liquid extraction procedure with MEOH. The retention times for AB and the internal standard were reported to be 4.9 ± 0.8 and 7.8 ± 1.2 min, respectively [20]. In our study, the molarity of disodium edetate and the pH were further increased by visual inspection of the peaks and the resolution was affected and improved with the mobile phase having a composition of acetonitrile–disodium edetate (20 mM) (45:55, v/v) at pH 4.5. Actually, the composition of the mobile phase in this latter case was same as described by Gondal et al. [24], in which plasma AB concentrations were determined by HPLC after extraction with MeOH, but detailed information about the resulting chromatograms and the retention times were lacking. Finally, in our experimental conditions reproducible chromatographic separations were achieved by increasing the pH of the mobile phase mixture of acetonitrile–edetate sodium (20 mM) (45:55, v/v) to 5.0. During the analysis UV detection was performed at 407 nm, which was observed to be suitable for both AB and the internal standard, although in some other studies detection at different wavelengths such as at 388 nm [18], 386 nm [19,20] or 382 nm [4,25,31] were mostly performed. Representative chromatograms of AB and the internal standard assayed in plasma were presented in Fig. 1. Typical chromatograms obtained for a blank plasma sample and for a plasma sample obtained from one of the cancer patients who had been treated with Ambisome® were shown Fig. 2. The retention times for AB and the internal standard in the standard working solutions and the plasma of patients were reproducible. The mean measured values for the retention times of AB and the internal standard were 6.27 ± 0.01 min (R.S.D. 1.53%, $n = 10$) and 3.77 ± 0.01 min (R.S.D. 1.11%, $n = 10$), respectively on different occasions. The retention time of AB found in our study was consistent with the retention time (approximately 6–7 min) reported by Wang et al. [31]. In our analysis, when the chromatograms obtained from the blank plasma, the plasma samples of the patients on antifungal therapy, and the plasma spiked with co-administered drugs, such as cyclophosphamide, daunorubucine, cisplatine, phenobarbital, diazepam, amikasin, ampicilline, acetaminophen, acetylsalicylic acid and chlorpromazine were examined, no endogenous compounds were observed at the retention times of both AB and the internal standard.
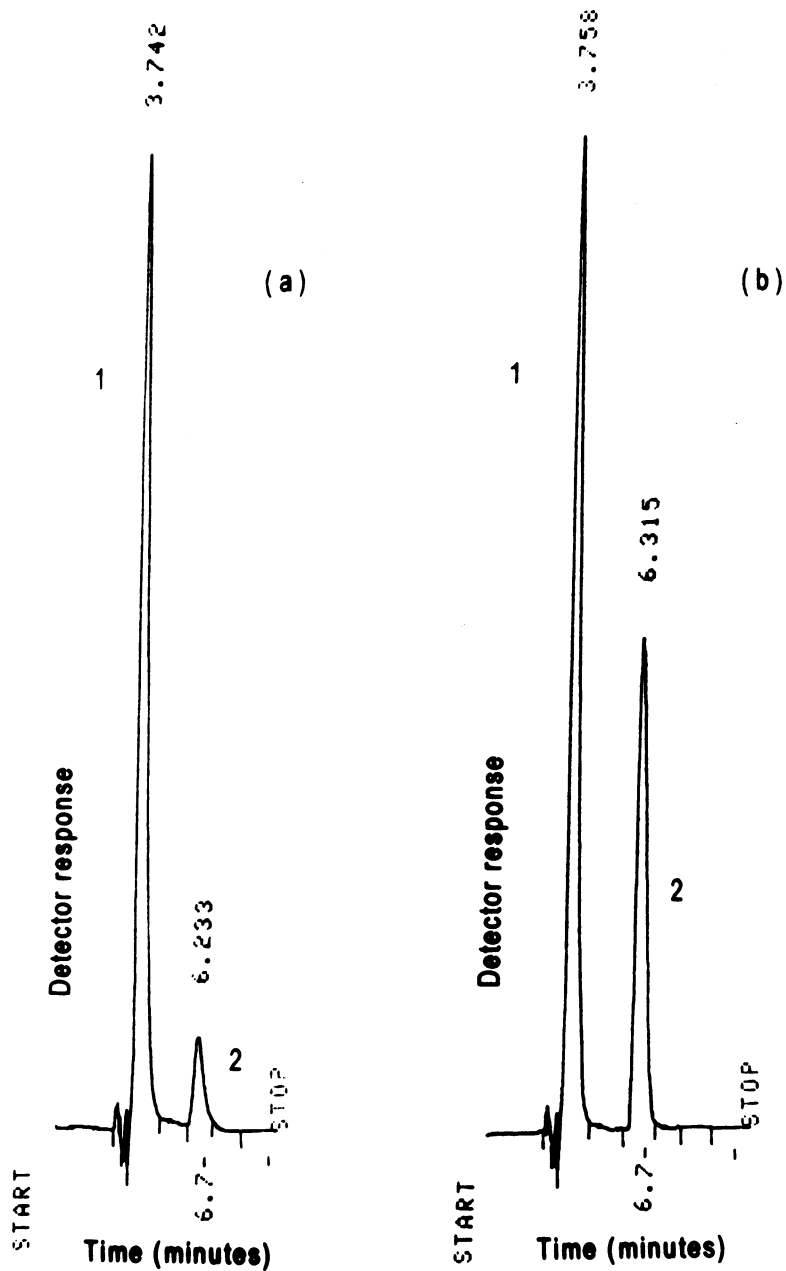
Fig. 1. Representative chromatograms of the working standard solutions of AB in plasma containing 0.100 μg ml^{-1} of AB (a) and 1.00 μg ml^{-1} of AB (b). (1) Internal standard (1-amino-4-nitronaphthalene); (2) Amphotericin B.

The standard curves of AB in plasma were linear over the specified range of 0.0100–2.00 μg ml^{-1}. A typical equation of the calibration curve relating the PHR of AB to the internal standard and the concentration of AB was $y = 1.00173x + 0.00127$ with a coefficient of determination of 0.9997 and the intercept was not significantly different from zero ($P = 0.05$). AB calibration

curves displayed good reproducibility for different days, and the mean of the slope, intercept and coefficient of determination were 1.0103 ± 0.00879 (R.S.D. 1.50%, $n = 3$), 0.00217 ± 0.000715 (R.S.D. 57%, $n = 3$), and 0.9995 ± 0.0000899 (R.S.D. 0.02%, $n = 3$), respectively. Among two different concentrations tested, the limit of quan-

titation defined as the lowest point of the calibration curve with acceptable accuracy and precision was 0.0100 µg ml^{-1}. The actual mean measured concentration of AB at this level was found to be 0.00997 ± 0.0002 µg ml^{-1} (R.S.D. 5.42%, $n = 5$). The limit of detection as defined in the experimental section was 0.005 µg ml^{-1}. In some of the
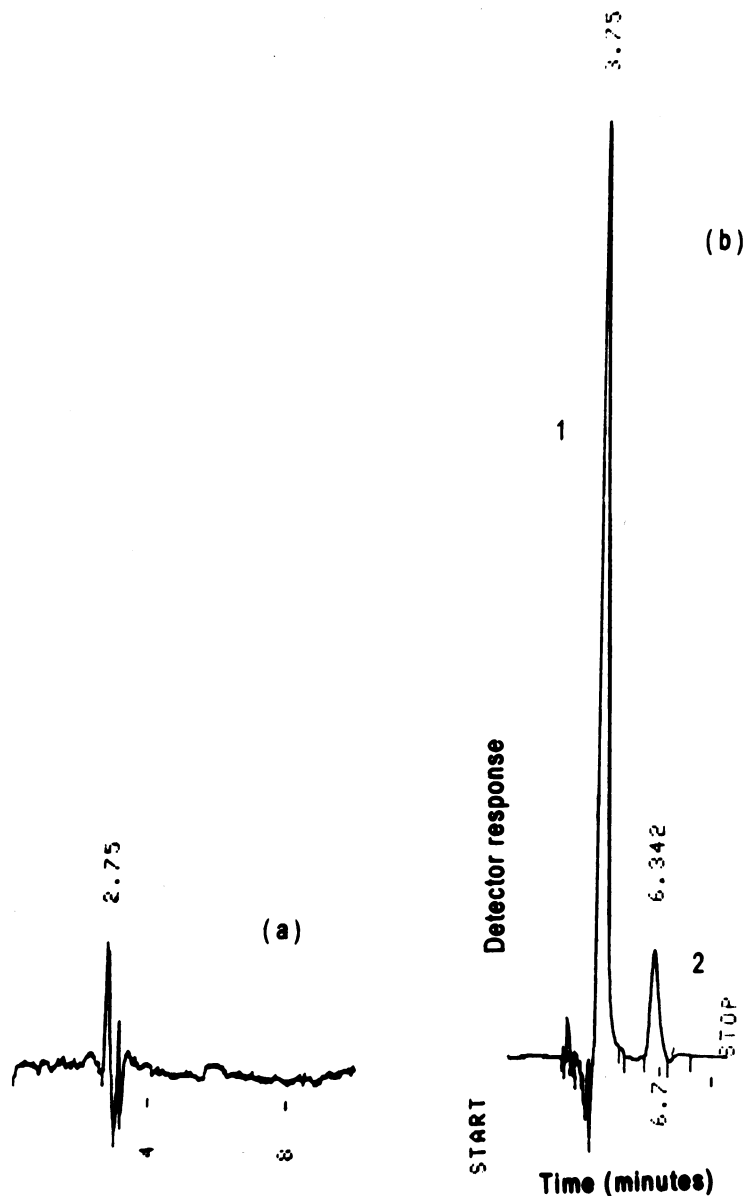

Fig. 2. Representative chromatograms of a blank plasma (a), and a patient's plasma showing trough levels of AB (139 ± 9 ng ml^{-1}) before i.v. infusion of the second dose of AmBisome® (b). (1) Internal standard (1-amino-4-nitronaphthalene); (2) Amphotericin B.

previous studies neither the quantitation limits nor its validation were reported, whereas only detection limits were given as sensitivity [19,20,31]. In this respect, the sensitivity of AB assay in plasma reported by Wang et al. [31] was also 0.005 µg ml^{-1}. The calibration range used in their study was between 0.005 and 2.50 µg ml^{-1}, but the accuracy at the lowest point over the calibration range was not demonstrated. Instead of that, 0.025 µg ml^{-1} of AB was regarded as the lowest concentration in the calibration range and its validation was performed. On the other hand, the sensitivity of the method reported by Gondal et al. was 0.01 µg ml^{-1} for AB without making an identification [24]. For this reason, in our study the limit of quantitation and detection were definitely described and an improvement in the quantitation limit compared with previously published studies [19,20,24,31] was demonstrated.

A summary of the results on precision derived from the measured concentrations of the working standard solutions in plasma at three different concentrations were demonstrated in Tables 2 and 3, respectively. The within-day R.S.D. were less than 2% at all concentrations and the day-to-day R.S.D. for 45 measurements was found to be 6.54%. These results clearly indicated the reproducibility and repeatability suited the purpose of the analytical method.

During the construction of calibration curves and the analysis of the samples, quality control plasma samples at three concentrations were employed. The results dealing with the triplicate analysis of three quality control samples at different days ($n = 27$) were demonstrated in Table 4. The R.S.D. values for all measurements on within-day and day-to-day basis were less than 15% with an overall R.S.D. of 4.88% for 27 measurements at three different days. Conse-

Table 2
Within-day precision of the method for analysis of AB in spiked plasma samples

Concentration (µg ml^{-1})	Added (%)	Found[a] (%)	Recovery[b] (%)	R.S.D. (%)
0.0200	2	2.00 ± 0.01	100 ± 1	1.20
1.00	100	98.3 ± 0.6	98.3 ± 0.6	1.34
1.75	175	172 ± 1	98.4 ± 0.8	1.83
Mean ± S.D., $n = 15$			99.0 ± 0.4	1.65
95% confidence limits			$100 > \mu > 98.1$	

[a] Calculated from peak height ratio (AB/IS) and expressed as found amount in percent, mean ± S.D., $n = 5$.
[b] Calculated from peak height ratio (AB/IS) and expressed as recovery in percent, mean ± S.D., $n = 5$.

Table 3
Day-to-day precision of the method for analysis of AB in spiked plasma samples

Concentration (µg ml^{-1})	Added (%)	PHR[a] (Day 1)	PHR[a] (Day 2)	PHR[a] (Day 3)	Found[b] (%)	Recovery[c] (%)	R.S.D. (%)
0.0200	2	0.0202 ± 0.0001	0.03 ± 0.01	0.0225 ± 0.001	1.85 ± 0.08	91.4 ± 3.7	8.92
1.00	100	0.990 ± 0.007	1.01 ± 0.45	0.970 ± 0.433	97.9 ± 1.5	97.9 ± 1.5	3.42
1.75	175	1.73 ± 0.01	1.82 ± 0.81	1.73 ± 0.77	175 ± 4	100 ± 1	2.29
Mean ± S.D., $n = 45$						96.6 ± 0.9	6.54
95% confidence limits						$98.5 > \mu > 94.7$	

[a] Peak height ratio (AB/IS), mean ± S.D., $n = 5$.
[b] Calculated from PHR and expressed as found amount in percent, mean ± S.D., $n = 15$.
[c] Calculated from PHR and expressed as recovery in percent, mean ± S.D., $n = 15$.

quently, during the measurements of plasma AB trough concentrations of the patients these results were taken into consideration and the concentrations measured during the runs were accepted. The plasma AB trough concentration of the first patient who received the first dose of AmBisome® by i.v. infusion was found to be 139 ± 9 ng ml^{-1} (R.S.D. 13.3%, $n = 4$) (Fig. 2b). The second patient was on antifungal therapy with AmBisome® for several days and the plasma AB trough concentration was 409 ± 11 ng ml^{-1} (R.S.D. 5.46% $n = 4$) at the end of 19 days.

Furthermore, during our preliminary studies and the course of the study it has been found that AB in plasma decomposes over time, when the samples were exposed to light. Although the stability of AB in various solutions and biological matrix at different temperatures were examined in some of the previous studies [2,19,31,33,39], the effect of light on AB degradation was not reported. In our study, special attention was given during the preparation and storage conditions of the samples and standard solutions and all of the

procedures including the SPE, till analysis by HPLC were carried out in the dark room. The results dealing with the light and temperature sensitivity of AB in plasma were demonstrated in Table 5. The effect of light and temperature on degradation of AB at 25°C was rapid and this degradation could be prevented by protecting the samples from the light exposure. In our study no change in AB concentration was detected with the plasma samples stored in the dark at $-20°C$ for 30 days.

4. Conclusion

A sensitive and selective HPLC assay was developed for AB quantitation by using the SPE. The SPE was demonstrated to have sufficient and reproducible extraction efficiency for AB from 1 ml of diluted plasma and no endogenous interfering peaks were observed in the plasma of the patients and the blank plasma spiked with AB or other drugs. The addition of 1-amino-4-ni-

Table 4
Recovery of AB from the quality control samples at different days

Concentration (μg ml^{-1})	Day 1 recovery[a] (%)	Day 2 recovery[a] (%)	Day 3 recovery[a] (%)	Different days recovery[b] (%)
0.0150	96.8 ± 1.1 (1.93)	97.8 ± 4.5 (8.01)	93.5 ± 5.66 (10.5)	96.1 ± 2.21 (6.88)
1.00	103 ± 3 (5.11)	97.9 ± 1.1 (1.97)	99.7 ± 1.2 (2.17)	100 ± 1 (3.63)
1.80	99.3 ± 0.4 (0.666)	99.7 ± 1.2 (2.17)	102 ± 0 (1.87)	99.8 ± 0.8 (2.35)
Mean ± S.D., R.S.D. (%), $n = 27$				98.7 ± 0.927 (4.88)

[a] Calculated from PHR and expressed as recovery in percent (mean ± S.D., R.S.D. (%), $n = 3$).
[b] Calculated from PHR and expressed as recovery in percent (mean ± S.D., R.S.D. (%), $n = 9$).

Table 5
Stability of amphotericin B in human plasma

Time (day)	% Drug remained[a]			
	25°C (light exposed)	25°C (in the dark)	4°C (in the dark)	-20°C (in the dark)
0	100 ± 0 (2.12)	100 ± 0 (2.12)	100 ± 1 (2.11)	100 ± 1 (2.12)
1	99.5 ± 1.0 (2.40)	99.9 ± 1.0 (2.39)	100 ± 2 (3.12)	100 ± 1 (2.18)
10	97.5 ± 1.0 (1.83)	98.5 ± 1.0 (1.83)	100 ± 1 (1.10)	100 ± 1 (1.51)
30	85.6 ± 0.3 (0.454)	91.6 ± 0.3 (0.670)	99.5 ± 0.1 (0.218)	100 ± 1 (1.78)

[a] Calculated from PHR and expressed as the percent drug remained (mean ± S.D., R.S.D. (%), $n = 3$).

tronaphthalene as the internal standard during the last elution step in the SPE procedure enhanced the reliability and flexibility of the HPLC assay for AB and improved the performance characteristics of the analytic parameters in the validation procedure. The protocol of the assay validation was explained in detail and fulfilled the necessary requirements, in which the stability of the plasma samples, the recovery, linearity, precision, accuracy and limit of quantitation of the described method were shown to have satisfactory results. Consequently, this method can be used in the research and clinical laboratories, either for AB therapeutic monitoring in plasma or single/multiple-dose pharmacokinetic analysis of AB in plasma of the patients that are hospitalized for antifungal therapy with the liposomal and other new lipid-based formulations of AB, since it offers a rapid chromatographic analysis time and can be performed on readily available equipment for routine use.

Acknowledgements

References

[1] D.W. Warnock, J. Antimicrob. Chemother. 28 (Suppl. B) (1991) 83–91.
[2] A. Adedoyin, J.F. Bernardo, C.E. Swenson, L.E. Bolsack, G. Horwith, S. Dewit, E. Kelly, J. Klastersky, J.P. Sculier, D. Devaleriola, E. Anaissie, G. Lopez-Berestein, A. Llanos-Cuentas, A. Boyle, R.A. Branch, Antimicrob. Agents Chemother. 41 (1997) 2201–2208.
[3] J.D. Cleary, S.W. Chapman, T.C. Hardin, M.G. Rinaldi, J.L. Spencer, J. Deng, G.J. Pennick, C.J. Lobb, Ann. Pharmacother. 31 (1997) 39–44.
[4] L.H. Wang, R.M. Fielding, P.C. Smith, L.S.S. Guo, Pharm. Res. 12 (1995) 275–283.
[5] L.H. Hanson, D.A. Stevens, Antimicrob. Agents Chemother. 36 (1992) 486–488.
[6] P. Villani, M.B. Regazzi, R. Maserati, P. Viale, F. Alberici, R. Giacchino, Arzneim.-Forsch/Drug Res. 46 (1996) 445–449.
[7] W. Mills, R. Chopra, D.C. Linch, A.H. Goldstone, Br. J. Heamatol. 86 (1994) 754–760.
[8] O. Ringden, J. Tollemar, Mycoses 36 (1993) 187–192.
[9] N. Collette, V. Der Auwera, F. Meunier, C. Lambert, J.P. Sculier, A. Coune, J. Antimicrob. Chemother. 27 (1991) 535–548.
[10] V.L. Kan, J.E. Bennett, M.A. Amantea, M.C. Smolskis, E. McManus, D.M. Grasela, J.W. Sherman, J. Infect. Dis. 164 (1991) 418–421.
[11] S.W. Sanders, K.N. Buchi, M.S. Goddard, J.K. Lang, K.G. Tolman, Antimicrob. Agents Chemother. 35 (1991) 1029–1034.
[12] F. Meunier, H.G. Prentice, O. Ringden, J. Antimicrob. Chemother. 28 (Suppl.B) (1991) 83–91.
[13] T.K.C. Ng, R.C.Y. Chan, F.A.B. Adeyemi-Doro, S.W. Cheung, A.F.B. Cheng, J. Antimicrob. Chemother. 37 (1996) 465–472.
[14] N. Cellat, Liposomal Amphotericin B (AmBisome®) in Antifungal Chemotherapy and its Pharmacokinetic Properties, Hacettepe University, Institute of Health Sciences, Ankara, 1995 M.Sci. Thesis.
[15] I. Nilsson-Ehle, T.T. Yoshikawa, M.C. Schotz, L.B. Guze, J. Infect. Dis. 135 (1977) 414–422.
[16] D.W. Warnock, M.D. Richardson, A. Turner, J. Antimicrob. Chemother. 10 (1982) 467–478.
[17] C.L. Golas, C.G. Prober, S.M. MacLeod, S.J. Soldin, J. Chromatogr. 278 (1983) 387–395.
[18] J.W. Mayhew, C. Fiore, T. Murray, M. Barza, J. Liq. Chromatogr. 274 (1983) 271–279.
[19] P.R. Bach, Antimicrob. Agents Chemother. 26 (1984) 314–317.
[20] G.G. Granich, G.S. Kobayashi, D.J. Krogstad, Antimicrob. Agents Chemother. 29 (1986) 584–588.
[21] C. Brasinne, C. Laduron, A. Coune, J.P. Sculier, C. Hollaert, N. Collette, F. Meunier, J. Chromatogr. 419 (1987) 401–407.
[22] L.H. Wang, P.C. Smith, R.M. Fielding, Pharm. Res. 7 (1990) S33.
[23] K.M. Wasan, K. Vadiei, G. Lopez-Berestein, D.R. Luke, J. Infect. Dis. 161 (1990) 562–566.
[24] J.A. Gondal, R.P. Swartz, A. Rahman, Antimicrob. Agents Chemother. 33 (1989) 1544–1548.
[25] R.M. Fielding, P.C. Smith, L.H. Wang, J. Porter, L.S.S. Guo, Antimicrob. Agents Chemother. 35 (1989) 1208–1213.
[26] R.T. Profitt, A. Satorius, S.M. Chiang, L. Sullivan, J.P. Adler Moore, J. Antimicrob. Chemother. 28 (Suppl. B) (1991) 49–61.

[27] J.W. Hülsewede, H. Dermoumi, Mycoses 37 (1994) 17–21.

[28] W.J. Lee, M.A. Amantea, P.A. Francis, E.E. Navarro, J. Bacher, P.A. Pizzo, T.J. Walsh, Antimicrob. Agents Chemother. 38 (1994) 713–718.

[29] K.M. Wasan, V.B. Grassie, Jr, G. Lopez-Berestein, Antimicrob. Agents Chemother. 38 (1994) 2224–2226.

[30] P. Longuet, V. Joly, P. Amirault, N. Seta, C. Carbon, P. Yeni, Antimicrob. Agents Chemother. 35 (1991) 1303–1308.

[31] L.H. Wang, P.C. Smith, K.L. Anderson, J. Chromatogr. 579 (1992) 259–268.

[32] V. Joly, R. Farinotti, L. Saint-Julien, M. Cheron, C. Carbon, P. Yeni, Antimicrob. Agents Chemother. 38 (1994) 177–183.

[33] M.P. Lambros, S.A. Abbas, D.W.A. Bourne, J. Chromatogr. 685 (1996) 135–140.

[34] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Meta. Pharmacokinetics 16 (1991) 249–255.

[35] ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodolgy, Step 4, 27 October 1994.

[36] L. Aarons, S. Toon, M. Rowland, J. Pharm. Methods 17 (1987) 337–346.

[37] S. Bolton, Pharmaceutical Statistics, Practical and Clinical Applications, second ed., Marcel Dekker, New York, 1990.

[38] The British Pharmacopoeia, The University of Printing House, Cambridge, 1993.

[39] L.C. Edmonds, L. Davidson, J.S. Bertino, Ther. Drug Monit. 11 (1989) 323–326.