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Therapeutic monitoring of amphotericin B in Saudi ICU patients using UPLC MS/MS assay

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ABSTRACT: Amphotericin B (AmB) is the first-line agent for the treatment of life-threatening invasive fungal infections. The aim of this study was to monitor AmB in critically ill Saudi patients in ICU after i.v. administration of 0.68 ± 0.1 mg/kg/day Fungizone®. A selective, sensitive and precise UPLC MS/MS method was developed to measure AmB concentrations in these patients. Seven ICU patients with creatinine clearance (ClCr) >40 mL/min were included. AmB levels were analyzed using a Waters Aquity UPLC MS/MS system, a BEH Shield RP_{18} column and detection via electrospray ionization source with positive ionization mode. The precision and accuracy of the developed UPLC method in the concentration range of 200–4000 ng/mL show no significant difference among inter- and-intra-day analysis ($p > 0.05$). Linearity was observed over the investigated range with correlation coefficient, $r > 0.995$ ($n = 6$/day). The pharmacokinetics of AmB in these patients, at steady state, showed a high terminal half-life of 124.6 ± 73.4 h, with a highest concentration of 513.9 ± 281.1 ng/mL, a lowest concentration 316.4 ± 129.0 ng/mL and a mean clearance 91.1 ± 39.2 mL/h/kg. The pharmacokinetics of AmB in critically ill Saudi patients in ICU was studied using a fully validated assay. A weak correlation ($r = -0.22$) of AmB Cl with ClCr was obtained, which suggests the need for further investigation in a larger population. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: amphotericin B; UPLC MS/MS; Fungizone®; pharmacokinetics; human

Introduction

Amphotericin B (AmB), a polyene antibiotic (Liu *et al.*, 1995), has been used as the 'gold standard' antifungal agent since 1960 (Gibbs *et al.*, 2005). AmB is the drug of choice in the treatment of life-threatening invasive fungal diseases in humans (US Food and Drug Administration, 2001; United States Pharmacopeia, 2007). AmB is amphipathic; that is, it has both hydrophilic and hydrophobic moieties that render it insoluble in water and most organic solvents. Aqueous solubility is achieved through formulation with sodium deoxycholate to produce a colloidal dispersion after reconstitution prior to intravenous infusion. AmB deoxycholate (Fungizone ®) is given to adults at 0.3–1.5 mg/kg/day. It is a highly potent antifungal formulation and inexpensive compared with other formulations on the market, but is associated with a high incidence of adverse effects, such as infusional toxicity and nephrotoxicity (Wingard *et al.*, 1999; Bates *et al.*, 2001).

Although it has been used widely in man for the past 50 years, there have been few well-designed pharmacokinetics studies in humans. AmB has a relatively short distribution phase and is best characterized with a two-compartment model for three of the studied patients, while a one-compartmental model is distinguished for the rest of patients. Since the plasma levels are determined 1 h after stopping the infusion, the one-compartmental open model is sufficient to explain AmB as justified as a vancomycin antibiotic in a clinical setting for simplicity (Bauer, 2006). It is highly bound to plasma proteins (>90%), primarily to lipoproteins, but it is poorly dialyzable. Little is known about AmB metabolism and distribution (Egger *et al.*, 2001). AmB elimination half-life could be as high as 15 days.

The primary route of elimination of AmB is not known (Drew, 2009). AmB concentrations in the cerebrospinal fluid rarely exceed 2.5% of those in the plasma. Despite introduction of newer antifungal agents for the treatment of systemic mycoses, AmB remains the standard treatment for many severe, invasive fungal infections. However, because of toxicities associated with its intravenous use, along with the expanded availability of safer treatment options, it is frequently reserved for patients who have severe, life-threatening invasive fungal infections or are unable to tolerate alternative antifungals (Drew, 2009). Early determinations of AmB were performed by microbiologic inhibition assays, which had low selectivity and precision and required long incubation periods to get the results (Thomas and Peters, 1977). Several different methods have been reported for quantitative determination of AmB in various biological fluids; these include second and third order-derivative ultraviolet

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Abbreviations used: AmB, amphotericin B; AUP, area under the peak; ESI, electrometry ionization; RT, retention time

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(UV) spectrometry (Italia *et al.*, 2009), Raman spectrometry (Ganière Monteil *et al.*, 1998) and colorimetry (Fournier *et al.*, 1998). High-pressure liquid chromatography (HPLC) methods for AmB have been described in serum, plasma, tissue (Granich and Kobayashi, 1986; Bekersky *et al.*, 1996; Polikandritou Lambros *et al.*, 1996; Echevarńa *et al.*, 1998; Zhan-rui *et al.*, 2009; Chakrabarty and Pal, 2011), respiratory secretions (Lopeza *et al.*, 1998) and pharmaceuticals (Zhan-rui *et al.*, 2009).

Recently, liquid chromatographic tandem mass spectrometry (LC/MS/MS) techniques with improved selectivity and sensitivity and able to overcome the effect of components in plasma, such as bilirubin, have been introduced for AmB in different biological fluids (Gideon *et al.*, 1988; Granich and Kobayashi, 1986; Heinemann *et al.*, 1997; Gibbs *et al.*, 2005; Hong *et al.*, 2007). However, these methods show lack of sensitivity, with minimum detection limits of 2 μg/mL in plasma (Xiong *et al.*, 2009; Deshpande *et al.*, 2010) , require longer run times of ~5.0 min (Lee *et al.*, 2001; Xiong *et al.*, 2009; Deshpande *et al.*, 2010) or require a complicated extraction procedure (Lee *et al.*, 2001).

The clinical use of AmB is limited by its severe toxicity, mainly hemolysis and nephrotoxicity (Bekersky *et al.*, 2002). Therefore, therapeutic monitoring of AmB is essential in patients in intensive care. Although the LC/MS/MS techniques are characterized by a faster run time, to our knowledge, no published data report an assay of AmB with retention time <3.5 min with simple and efficient extraction techniques. The present study was undertaken to develop a fast, robust, selective, sensitive and precise UPLC MS/MS method for monitoring AmB in human plasma using clopidogrel as IS. Using this method the pharmacokinetics of AmB in ICU patients was examined after intermittent intravenous infusion administration of 0.51−0.75 mg/kg of Fungizone®.

Experimental

Chemicals and reagents

Amphotericin-B (99.8% purity), clopidogrel and formic acid were purchased from Sigma–Aldrich (St Louis, MO, USA). All other reagents and chemicals were of HPLC analytical grade, and were used as received. Water was deionized and purified using a Milli-Q Reagent Grade water system (Millipore Corporation, Bedford, MX 01730, USA).

Study design

A prospective cross-sectional study was conducted at Prince Sultan Military Hospital in Riyadh, Saudi Arabia. After the approval of the study by the Military Hospital Research and Ethics Committee, the patients were recruited with interned consent. Detailed information from eligible patients was collected. The participants had received Fungizone® for at least 7 days to ensure a steady-state plasma level of AmB. Patients were excluded from the study if their creatinine clearance (ClCr) was <40 mL/min, they required daily monitoring of their serum creatinine and drug plasma level, or they were receiving the IS.

Patients and sampling time

Seven ICU patients with ClCr > 40 mL/min were included in the study. After diagnosis the patients were given 0.68 ± 0.10 mg/kg/day of AmB via intermittent intravenous infusion over 0.5 h for at least 7 days to ensure steady state. Fungizone® formulation of AmB is prescribed at Prince Sultan Military Hospital in Saudi Arabia for fungal infection treatment. Blood samples (5 mL) from each patient were collected in heparinized tubes at 0, 1, 2, 4, 8, 12 and 24 h after drug administration. Plasma samples were separated by centrifugation at 4000 rpm for 15 min and stored at −20°C prior to assay. Demographic data (age, weight, height and sex) and plasma creatinine concentration were collected from patient medical records as shown in Table 1.

Chromatographic conditions

Analysis was carried out on a Waters Acquity UPLC™ system with autosampler and column manager oven. An Acquity UPLC™ BEH Shield RP_{18} (1.7 μm column, 2.1 × 50 mm, Waters Corp, Milford, MA,USA) was used for separation with column temperature was maintained at 40°C. For chromatographic optimization, different mobile phases were examined at different pH using a variety of columns. AmB has two pK_a values, a basic pK_a (8.12) owing to an amino group at position 48 and an acidic pK_a (3.72) owing to an acidic group at position 15 (Deshpande *et al.*, 2010). Therefore, a pH of 3.0 ± 0.2, suitable for keeping AmB in one ionic state for MS analysis, was used. Ammonium formate (10 mM) was selected to avoid overloading of ionization source with buffer (Deshpande *et al.*, 2010). The gradient elution for UPLC analysis consisted of two solvent compositions: solvent A, methanol–acetonitrile (50:50 v/v) containing 0.1% formic acid; and solvent B, 10 mM ammonium formate (pH 3 ± 0.2), containing 0.2% formic acid and 1% acetonitrile. The gradient began with 35.0% eluent A and increased to 90% A over 2.0 min prior to return to 35.0% A at 2.7 min. Throughout the process the flow rate was set at 0.3 mL/min at the start of the run and increased to 0.4 mL/min within 2 min, returning to 0.3 mL/min at 2.7 min. The total run time was 3.2 min. Data were collected in multi-channel analysis mode and processed using MassLynx™ V 4.1 software with TargetLynx™ V 4.1 program (Waters Corp., Milford, MA, USA) for analysis.

The UPLC was connected to a triple quadropole tandem–mass detector (Water, Corp), with an electrometry ionization (ESI) source for mass spectroscopy detection. The ESI source was set in positive ion mode and quantification was performed using multiple-reaction monitoring mode for the most suitable mass transitions. The optimal mass spectrometry parameters are listed in Table 2. The most prominent

Table 1. Demographic data of the patients

Patient no.	Gender	Age	Height (cm)	Weight (kg)	SCr (mg/L)	Dose (mg) for amphotericin B i.v./24 h
1	F	65	152	68	0.47	40
2	M	18	178	88.8	0.97	60
3	M	76	166	72	0.66	50
4	M	81	167	90.3	0.64	60
5	F	68	162	79.4	1.06	60
6	M	73	185	98.3	0.64	50
7	M	79	179	80.5	0.70	60

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Table 2. Setting for MS/MS detection of amphotericin B (AmB)

Source (ESI +) and analyzer	Settings
Capillary voltage (kV)	3.5
Cone voltage (V)	15
Extractor (V)	2.0
Radio frequency lens (V)	0.10
Source temperature (°C)	115
Desolvation temperature (°C)	400
Cone gas flow (L/h)	6
Desolvation gas flow (L/h)	800
Collision energy (eV)	15
Collision gas flow (mL/min)	0.25

and stable fragments for AmB, Fig. 1, with scan time of 0.10 s per transition. Argon was used as the collision gas at a pressure of approximately 0.25 Pa. The optimized collision energies for AmB and IS were 15 and 37 eV, respectively.

Preparation of amphotericin B standards and quality control samples

All solutions of AmB were stored in polypropylene containers to minimize adsorption of AmB to glass (Lee *et al.*, 2001). Stock standard solution of AmB was prepared in methanol–DMSO (9:1, v/v) at a concentration of 0.2 mg/mL and stored in 4.0 mL plastic vials at −20°C covered with aluminum foil to protect AmB from light. For IS stock solution 5.0 mg was dissolved in 10 mL methanol and stored at −20°C. Different working standard solutions of AmB (200–4000 ng/mL) and the IS (1.5 μg/mL) were prepared by dilution of the above mentioned stock solutions in pure methanol and were kept at −20°C.

Plasma sample preparation

The plasma calibrations standards were prepared in six replicates at different concentrations between 200 and 4000 ng/mL in a 1.8 mL Eppendorf tube. Blank human plasma (250 μL) was spiked with aliquots of AmB working solutions and 20 μL (30 ng) of the IS. Low, medium and high concentration quality control (QC) samples at concentrations of 250, 1000 and 2000 ng/mL of AmB with 120 ng/mL of IS. The prepared plasma samples were subjected to protein precipitation by the addition of 1 mL of methanol; the mixture was vortexed at high speed for 1 min followed by centrifugation at 20,000 rpm for 15 min at 10°C. The supernatant was transferred into a clean 5 mL Pyrex glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 150 μL of 90% methanol solution. After vortexing

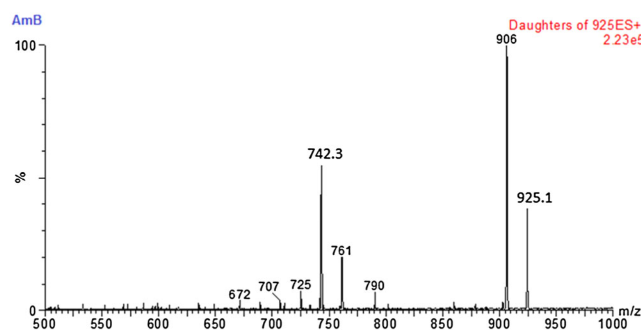

Figure 1. MS fragments of amphotericin B.

for 1 min, centrifuging at 4000 rpm for 4.0 min, and transferring into a glass autosampler vial with pre-slit septum (Waters, USA), 2.0 μL were injected into the UPLC MS/MS system for analysis.

Ion suppression study

The absence of ion suppression was demonstrated by the method of Matuszewski *et al.* (1998). Six different batches of drug-free and IS-free human plasma were extracted. The extracts were reconstituted with AmB at three nominal concentrations 250, 1000 and 2000 ng/mL (low, medium, high). The peak areas of the samples were compared with those of the unextracted reference standard solutions containing the equivalent nominal amount of AmB in the mobile phase (n = 6). The mean area ratio (reconstituted extracts/reference solutions) was 0.95 for AmB with RSD of <3.9%. Thus, no ion-suppression was observed.

Method validation

The developed method was validated in regards of selectivity, linearity, precision, accuracy, carryover, extraction recovery and stability in accordance with US Food and Drug Administration (2006) and United States Pharmacopeia (2007) guidelines. The selectivity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. Therefore, six blank, drug-free, plasma samples obtained from six donors were analyzed according to the procedure described above. The corresponding chromatograms were tested for possible interferences at the retention times (RT) of AmB and the IS.

The specificity of the method was investigated by comparing the chromatogram of blank plasma spiked with standard solutions to the samples collected from subjects after AmB administration. Intra-day precision and accuracy were determined within one day by analyzing six replicates of the QC samples at concentrations of 250, 1000 and 2000 ng/mL of AmB. The inter-day precision and accuracy were determined on three separate days. The intra- and inter-day precision were defined as the relative standard deviation (RSD). The accuracy was presented as percentage relative error, RE [(measured concentration − nominal spiked concentration)/nominal spiked concentration] × 100. Extraction efficiency was determined for the QC samples at the three concentrations levels (low, medium, high) with those of post-extraction spiked blank plasma samples for AmB concentrations. The absolute extraction recoveries were calculated by comparing the peak areas of the samples with those of the unextracted standard solutions containing the equivalent amount of AmB (n = 6). The nominal value of AmB concentration (ng/mL) in plasma was plotted as a function of the peak area ratios obtained of AmB and the IS. The day curve was accepted if the RSD was <20% for all the tested concentrations (low, medium and high).

The limit of detection (LOD) was defined as the lowest concentration of the analyte resulting in a signal-to-noise ratio of >5:1. The lower limit of quantitation (LLOQ) was defined as the lowest drug concentration of the analyte resulting in a signal-to-noise ratio >10, which can be determined with an RSD <20% and an accuracy of 100 ± 20% on a day-to-day basis.

Accuracy and precision at the LLOQ were estimated. The robustness of the method was determined using two different Acquity UPLC^{TM} BEH RP_{18}, 1.7 μm columns, 2.1 × 50 mm (Waters Corp, Milford, MA,USA).

Freeze–thaw stability of the plasma samples was evaluated by exposing quality control samples to three freeze (−20°C) and thaw (room temperature) cycles after 0, 72 h, 1 week and 2 weeks of preparation and storage at −20°C. The stability of the processed samples was assessed by keeping sample vial sealed with parafilm at 10°C. The samples were analyzed at 0, 24, 48 and 72 h after preparation.

AmB was also subjected to a drastic conditions, in amber volumetric glass, by diluting it in water, 1 M solution of NaOH and 2 M HCl solution (n = 6) and AmB area under the peak (AUP) was measured as the zero AUP. Each solution was carefully heated to boiling and left to cool down and AmB AUP after boiling was measured.

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Data and statistical analysis

All data in this study were expressed as either the mean ± SD of six replicates for the assay or seven replicates for the human study. The standard curves were calculated by linear regression without weighting, using the equation $y = a + bx$, where y is the AUP ratio of the drug to the IS, a is the intercept, b is the slope, and x is AmB concentration. The RSD was calculated for all values.

The Cockcroft–Gault equation was used [equation 1] to estimate creatinine clearance for males (ClCr, mL/min):

$$\mathrm{ClCr_{male}} = \frac{(140 - \mathrm{age})^{*}\mathrm{IBW}}{72^{*}\mathrm{SCr}} \quad (1)$$

where $\mathrm{ClCr_{female}} = 0.85\ \mathrm{ClCr_{male}}$, age = patient's age (years) and IBW = ideal body weight (kg). If a patient weighed ≤130% of IBW, actual body weights were used or adjusted body weight [AdjBW = IBW + 0.4 (patient weight, kg − IBW)].

The plasma AmB concentration vs time data of the patients were analyzed using a noncompartmental method for simplicity (Bauer, 2006), although the drug was given via intermittent intravenous infusion. The maximum concentration 1 h after completing the infusion is $C_{peak,ss}$ and the minimum concentration taken prior to the next intravenous infusion dose is $C_{trough,ss}$. Both $C_{peak,ss}$ and $C_{trough,ss}$ were obtained from participants. The elimination rate constant k was calculated from the slope of the regression line that best fit the terminal part (last three or four points) of the log–linear concentration–time curve. The dosing interval, τ, was 24 h in this study. The terminal half-life ($t_{1/2}$) was calculated from 0.693/k while the total body clearance (Cl) was estimated from dose/AUC_{0-24}, where AUC_{0-24} is the area under the curve from time 0 to 24 h (during the dosing interval) and was calculated using the trapezoidal rule. The volume of distribution (V) was determined from the equation $k = Cl/V$.

The Student t-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. Other tests were used using IBM SPSS Statistics 20. A p-value < 0.05 was considered significant.

Results

Chromatography

Mass spectroscopy (MS) parameters were optimized for achieving selectivity and sensitivity in positive ion mode. Both the analyte and internal standard (IS) have ability to accept protons and generate [M + H]+ ions. Protonated parent ions for AmB and IS quantification were achieved using multiple-reaction monitoring of the transitions of m/z 925.1 → 742.3 for AmB (Fig. 1) and m/z 321.8 → 155 for IS.

Figure 2 is a representative chromatograms of blank human plasma spiked with the AmB LLOQ (200 ng/mL) and IS (A1), and the patient plasma after 24 h after Fungizone® i.v. infusion administration is depicted in Fig. 2(A2). The optimum conditions eluted AmB and IS at 1.7 and 2.1 min, respectively, showing proper separation. The assay run time was 3.2 min, which is the shortest RT among published AmB separation assays (Xiong *et al.*, 2009; Deshpande *et al.*, 2010). During the 7 months of validation, there was no significant change in the observed RT of AmB or the IS (RSD < 0.4 %).

UPLC MS/MS validation

There was no endogenous plasma components eluted at the retention times of either AmB or the IS (Fig. 2) in any of the spiked blank plasma samples collected from different donors or from patients who received different classes of medications, which proves the assay specificity.

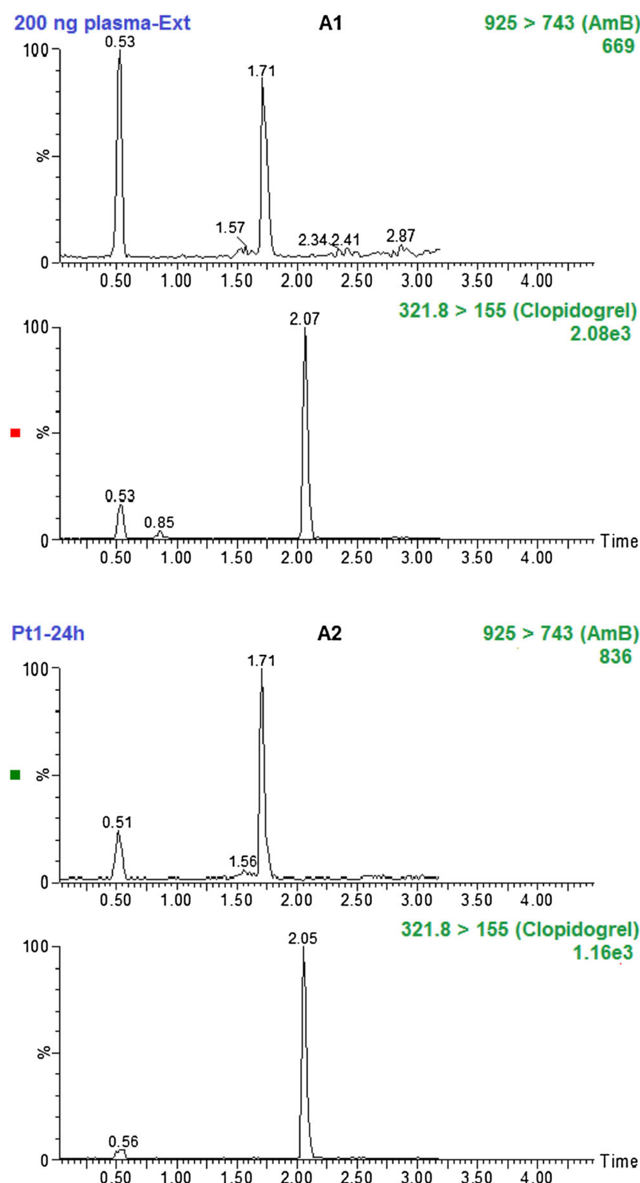

Figure 2. Multiple reaction monitoring transition of standard amphotericin B (AmB) at 200 ng/mL and IS and its transition (A1) and AmB chromatogram after 24 h of Fungizone® administration to patient 1and its transition (A2).

Excellent separation between AmB and IS with minimal background baseline noise was obtained (Fig. 2). It was also observed that the representative chromatogram of patient plasma samples showed similar chromatographic behavior to spiked or quality control samples and none of the other patients' medications interfered or interacted with AmB. These findings demonstrate good assay selectivity.

The LOD of this assay was 50 ng/mL in human plasma with the corresponding RSD of 20% at a signal-to-noise ratio of >5 while, the LLOQ was 200 ng/mL at a signal-to-noise ratio of >10, with the corresponding RSD of 14% at an injection volume of 2.0 μL (Fig. 2). The peaks of AmB and IS were well resolved and LLOQ was sensitive enough to detect AmB at all sample time points used (Fig. 2A1 and A2).

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Excellent linear relationships ($y = 0.0051x - 0.08$) with $r^2 > 0.993$ were demonstrated between the AUP ratio of AmB and the IS in human plasma over the studied concentration ranges. The mean r was >0.997 and the RSD of the slopes of the three standard plots was <13.3%. Analysis of variance of the data indicated no significant difference ($p > 0.05$) in the slopes, intra- and inter-day, of the calibration curves. The results confirmed the reproducibility of the assay method.

The mean percentage recovery of AmB was 93.7 ± 7.2 with an RSD of ≤8. There was no significant difference in the extraction efficacy of the present assay over the range of concentrations studied. The accuracy and precision results are shown in Table 3. Precision is represented as RSD and accuracy was calculated as relative error (RE). The intra- and inter-run precision (RSD) was <13% and accuracy as RE was <8%.

There was no evidence of sample carryover from run to run. In addition, the matrix effect assessed by spiking samples post-processing showed <8% difference from spiked injection solvent.

Figure 3 shows that AmB was stable in the processed samples held in the autosampler at 10°C for 24 h with mean calculated values within 6.7% of the nominal concentration. However, the samples lost 67.3% (RSD of 7.9%) of its nominal concentration within 3 days if stored protected from light in the autosampler. Therefore, it is not recommended to keep AmB in the autosampler for longer than overnight to ensure reproducibility of the assay. The freeze–thaw temperature cycles did not significantly ($p > 0.05$) affect the stability of AmB in first cycle, after 72 h, with the mean calculated values within 2.3% of the nominal concentration, while after the third cycle, 42% of AmB was lost with an RSD of 5.6%. Unexpectedly, exposing AmB to drastic conditions revealed that AmB is stable in 2 M HCl solution even after boiling, losing only 5.7% (RSD of 7.3%) of its nominal value.

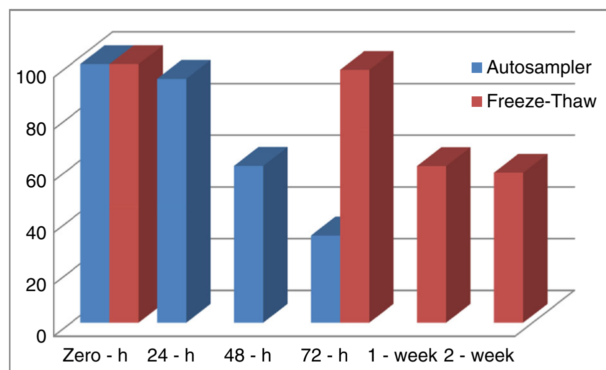

Figure 3. Stability of amphotericin in autosampler and after freezing–thaw cycles for 2 weeks ($n = 6$).

Table 3. Inter- and Intra-day statistics

Drug		Measured concentration (ng/mL)		
		Nominal concentration (ng/mL)		
		250	1000	2000
	Day 1	285	1190	2126
		215	881	1874
		210	1450	2240
		290	855	1760
		295	1110	2148
		205	900	1874
	Day 2	225	1080	2100
		237.5	992	1900
		262.5	900	1640
		275	1250	1700
		232.5	1300	2300
		235	800	2100
	Day 3	205	1200	1850
		280	1100	2020
		200	1000	2150
		230	850	2200
		270	900	1800
		260	1080	1950
Inter-day statistics	n	6	6	6
	Mean	245.1	915.7	1826.6
	SD	32.3	150.9	195.6
	Accuracy (RSD%)	17.6	14.2	9.6
	Precision (CV%)	13%	16.5	10.7
Intra-day (on day 3), $n = 6$	N	18	18	18
	Mean	240.8	1021	2018
	SD	34.1	131.2	162.1
	Accuracy (RSD%)	14.1	12.8	8.03
		−18	1.85	0.183

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In the meantime, both water and 1 M NaOH showed complete loss of AmB even without boiling for all tested samples (n = 6).

Amphotericin B pharmacokinetics in Saudi critically ill patients

The present study was conducted on seven ICU patients who were given Fungizone® (0.68 ± 0.10 mg/kg/day) for at least 7 days to ensure steady-state conditions. Plasma concentration–time profiles of AmB after dosing by i.v. infusion are depicted in Fig. 4. The decline of AmB concentrations in three patients suggested a two-compartment pharmacokinetics open model (fast distribution and slow elimination), while the remaining four patients suggest a single phase described by one-compartment pharmacokinetics open model. Since one- and two-compartment models were being obtained among patients and determining the AUC more than predicting the hourly concentration was the main concern of the present work, a noncompartmental model was selected to describe AmB pharmacokinetics in these patients, as presented in Table 4. The observed mean $C_{peak,ss}$ was 513.9± 281.1 µg/L. AmB showed a long elimination $t_{1/2}$ of from 2.3 to 9.7 days with a slow clearance of 91.1 ± 39.2 mL/h/kg, and the V was 18.9 ± 16.9 L/kg. The patients' creatinine clearance were plotted against their calculated AmB clearance, as shown in Fig. 5. There is a weak correlation (r = −0.22) between creatinine clearance and AmB clearance in these patients.

It should be mentioned that the developed assay, with no modification, was also applied successfully to measurement of AmB in rat plasma after AmB oral delivery system administrations (unpublished data).

Discussions and conclusion

A simple, rapid and sensitive analytical method was developed and validated for the analysis of AmB in human plasma. One of the advantages of this method is the use of a plasma protein precipitation procedure for sample preparation, which is less expensive than solid-phase extraction, and less time consuming. Moreover, the method requires considerably less plasma, which is important for pharmacokinetic studies involving repeated blood sampling. The chromatographic runtime is also short. Therefore, the developed bioanalytical method can be reliably employed as an assay method for pharmacokinetic study of any dosage form containing AmB in human.

Fungizone pharmacokinetic parameters in Saudi critically ill patients were compared with published data for the same formulation as shown in Table 5 (Bekersky *et al.*, 2002; Ayestarán

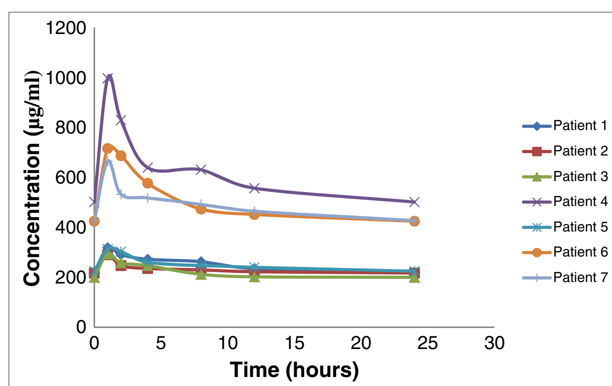

Figure 4. Steady-state amphotericin B plasma concentrations time profiles in critically ill Saudi patients after 0.68 ± 0.10 mg/kg/day as intermittent i.v. infusion of Fungizone® (n = 7).

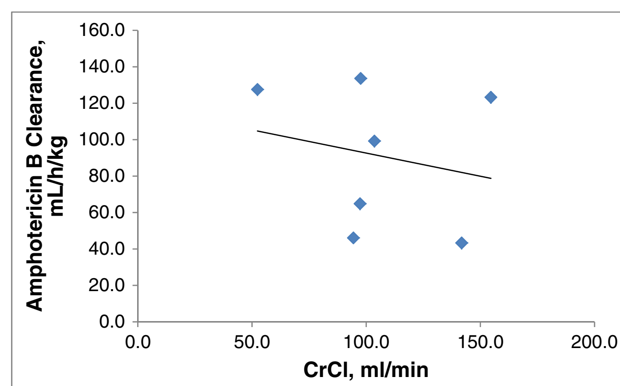

Figure 5. Relationship between the values of creatinine clearance and amphotericin B clearance in critically ill patients.

Table 4. Pharmacokinetic parameters of amphotericin B at steady state given as intermittent infusion every 24 h to Saudi critical ill patients (n = 7)

Parameters	Patient number							Overall		
	1	2	3	4	5	6	7	Mean	SD	RSD%
ClCr, mL/min	103.6	154.6	97.6	94.4	52.4	141.8	97.3	106.0	33.7	31.8
Dose, µg/kg	588.2	675.7	694.4	664.5	755.7	508.7	745.3	661.8	87.5	13.2
C_{peak}, ng/mL	319.1	290.5	294.0	997.5	313.9	716.5	665.8	513.9	281.1	54.7
C_{trough}, ng/mL	217.9	217.5	200.3	501.8	224.8	425.0	427.3	316.4	129.0	40.8
$t_{1/2}$, h	65.9	220.9	232.9	54.3	115.6	108.7	73.4	124.6	73.4	59.0
AmB Cl, mL/h/kg	99.3	123.3	133.6	46.1	127.6	43.3	64.9	91.1	39.2	43.1
V, L/kg	9.4	39.3	44.9	3.6	21.3	6.8	6.9	18.9	16.9	89.5

ClCr, Creatinine clearance; C_{peak}, maximum concentration 1 h after completing the infusion; C_{trough}, minimum concentration taken prior to the next intravenous infusion dose; $t_{1/2}$, terminal elimination half-life; Cl, total clearance; V, volume of distribution.

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Table 5. Comparative pharmacokinetics (mean ± SD) of AmB in human after Fungizone® administration (from 1998 to present)

Study	Assay method	Human demography status	Dose (mg/kg/day)	$C_{peak,ss}$ (μg/mL)	$t_{1/2}$ (h)	V (L/kg)	Cl (mL/h/kg)
Present	LC/MS/MS	Critically ill patients (immunecompromised; n = 7)	0.66 ± 0.09	0.51 ± 0. 28	124.6 ± 73.4	18.9 ± 16.9	106.0 + 33.7
Bekersky *et al.* (2002)	HPLC -LC/MS/MS	Healthy volunteer (n = 22)	0.6	1.43 ± 0.2	127 ± 30	NA	13.1 ± 2.0
Heinemann *et al.* (1997)	HPLC	Presumptive or proven fungal infection (n = 11)	1.0	1.78 ± 0.25	25.83 ± 9.4	2.61 ± 1.35	61.8 ± 33.6
Bassetti *et al.* (2011)	HPLC	Invasive fungal infections in immune-compromised patients (review)	0.6	1.06	91.1	5.1	34.1
Weiler *et al.* (2011)	HPLC	Critical ill patient (n = 2)	5	1.13	29.5	4.5	105
Weiler *et al.* (2012)	HPLC	Critical ill patient (n = 11)	3.7 + 2.68	1.24 ± 0.53	19.56 ± 8.44	4.65 ±2.01	190 ± 130
Ayestarán *et al.* (1996)	HPLC	Patient with neutropenia (n = 16)	1.0 in IL 20%	1.46 ± 0.61	11.44 ± 5.18	1.04 ± 0.51	62.97 ± 35.51
			1.0 in 5% D	2.83 ± 1.17	0.64 ± 0.24	0.56 ± 0.15	33.01 ± 14.33

$t_{1/2}$, Terminal elimination half-life; Cl, total clearance; V, volume of distribution; NA, not available; IL, intralipid; D, dextrose.

et al., 1996; Heinemann *et al.*, 1997; Bassetti *et al.*, 2011; Weiler *et al.*, 2011, 2012). It should be mentioned that, although the literature shows few pharmacokinetic studies characterized by insufficient number of patients or blood sampling points, a wide range of patient age involved or various techniques of drug assays used, there are some agreements with the present study. After Fungizone® administration, the mean reported clearance of AmB varied among the literature from 13.1 (Bekersky *et al.*, 2002) to 190 mL/h/kg (Weiler *et al.*, 2012). AmB Cl in Saudi patients in the present study is 91.1 ± 39.2 mL/h/kg, which is in agreement with most published values (Heinemann *et al.*, 1997; Weiler *et al.*, 2011, 2012; Ayestarán *et al.*, 1996), but is in disagreement with other published data (Heinemann *et al.*, 1997; Bekersky *et al.*, 2002; Bassetti *et al.*, 2011; Weiler *et al.*, 2011, 2012) which represent either unusually narrow range values of Cl (13 ± 2.0 mL/l/kg) in 22 volunteers or no range of Cl. However, the reported V in this study (2.5–34.1 L/kg) was greater than most reported values. This could be due to the high protein binding characteristic of AmB, which could have different degrees of binding affinity among patients (Heinemann *et al.*, 1997). In the present study AmB $t_{1/2}$ is in agreement with the higher values in the literature (Bekersky *et al.*, 2002). This could be explained by the method used to determine the half-life among studies and the reported lower values of clearance with higher distribution.

Although in this study patients were given conservative doses of AmB compared with Bassetti *et al.* (2011), the observed $C_{peak,ss}$ values were lower than all the reported data. The $C_{peak,ss}$ variabilities (30–118% difference) could be explained by the different metabolic properties of the respective patients, as reported by Tollemar and Rengden (Heinemann *et al.*, 1997), the disease status of the patients or the wide distribution of the drug in these patients.

This study investigated the existence of any trend of agreement between the dug clearance and the patient creatinine clearance, as demonstrated in Fig. 5. It shows a trend but with weak correlation. It remains unclear to what extent these clinical data may be explored in the clinical situation in humans with different ClCr, and a large number of healthy patients are needed to investigate this trend.

This study extends previous observations in healthy subjects (Bekersky *et al.*, 2002), neutropenic patients (Ayestarán *et al.*, 1996) and patients with disseminated fungal infections (Heinemann *et al.*, 1997; Bassetti *et al.*, 2011) that AmB pharmacokinetics are multiphasic, the drug is distributed widely to tissues (Weiler *et al.*, 2012) and it has a long elimination half-life and a total clearance substantially lower than the plasma flow to any major organ, as reported by Bekersky *et al.* (2002).

The developed assay was successfully applied to patients in intensive care and followed their time profiles within a dosing interval of almost a steady-state condition. It should be mentioned here that there was no interference from any administered drugs other than AmB on its determination using the condition described. Although the number of patients in this study was limited, the data indicated some important results for future studies, such as the relationship between ClCr and AmB clearance and a larger AmB volume of distribution among studied Saudi patients. Further limitations of this study comprise the heterogeneity of underlying diseases. The differences in the pharmacokinetics within the patients could be due to inter-individual variability of the patient protein binding (highly protein bound) and/or metabolism.

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Conflict of interest

The authors have no conflict of interests to disclose other than what has been acknowledged above.

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