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Determination of free and liposomal Amphotericin B in human plasma by liquid chromatography–mass spectroscopy with solid phase extraction and protein precipitation techniques

Niranjan M. Deshpande [a,b,*], Manish G. Gangrade [b], Maharudra B. Kekare [a], Vikas V. Vaidya [c]

[a] *Department of Chemistry, Kirti M. Doongursee College, Mumbai University, Dadar, Mumbai 400 028, India*
[b] *Cipla Ltd., Vikhroli, Mumbai 400 083, India*
[c] *Department of Chemistry, Ramnarain Ruia College, Mumbai University, Matunga, Mumbai 400 019, India*

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ABSTRACT

Amphotericin B is available in various drug delivery systems such as cholesteryl sulfate complex, as lipid complex, and as liposomal formulation. The separation and measurement of free drug (drug which is not bound with liposomal lipids) and liposomal drug (drug which is entrapped in liposomes) in the human plasma after injection of liposomal Amphotericin B is of prime importance due to toxicity concerns. A robust, specific and sensitive method has been developed to effectively separate and then quantify the free drug and liposomal drug, present in human plasma. This method utilizes solid phase extraction Oasis HLB cartridges, which retains the free drug and the liposomal Amphotericin B was eluted from the cartridge in first step. The eluted liposomal Amphotericin B was then extracted from lipids by protein precipitation method using 2% dimethylsulfoxide (DMSO) in acetonitrile. After separation and extraction, the quantification of free and liposomal fractions of Amphotericin B was performed by HPLC–MS–MS technique. The chromatographic separation was performed using Chromolith Performance RP 18e column. The mobile phase composed of 5 mM ammonium acetate, methanol and acetonitrile and a gradient elution program was used. The calibration curves were found to be linear for free Amphotericin B (0.25–15.0 μg/ml) and liposomal Amphotericin B (1.0–100.0 μg/ml). The recovery was about 96% for free Amphotericin B and about 92% for liposomal Amphotericin B. Recoveries were consistent over the linearity ranges defined. The intra-batch and inter-batch accuracy and precision fulfilled the international requirements. The stability of free and liposomal Amphotericin B was assessed under different storage conditions.

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1. Introduction

Systemic disseminated fungal infections are major cause of mortality and morbidity in patients with leukemia receiving chemotherapy and in a variety of immuno-deficiency. As the few drugs used in treatment of fungal infections are extremely toxic to the host, the treatment is challenging and hence it is required to give lowest possible dose. Since the drugs are diluted in blood and significant amount of drugs may get degraded or excreted or taken up by uninfected tissues, large doses are required to be administered for effectiveness of treatment, which has limitations due to toxicity of such drugs [1]. Amphotericin B (Fig. 1a) is a macrocyclic, polyene, antifungal antibiotic produced from a strain of *Streptomyces nodosus* that is widely used for the treatment of systemic fungal infections. It is fungistatic and fungicidal depending on the concentration of the drug in various body fluids and the susceptibility of specific fungus [2,3]. The majority of these infections are caused by the species of *Candida* and *Aspergillus* [2,4]. Amphotericin B acts by binding to ergosterol, the sterol component of the fungal cell membrane, leading to alterations in cell permeability and promoting the leakage in of other cell substances and subsequent cell death [2]. While Amphotericin B has a higher affinity for the ergosterol component of the fungal cell membrane, it can also bind to the cholesterol component of the mammalian cell leading to numerous toxic effects. Most patients receiving Amphotericin B intravenously experience acute infusion related toxicity, such as fever, chills, rigors, hypotension, nausea, vomiting, headache and thrombophlebitis. It also produces dose limiting complications like nephrotoxicity [5]. The use of liposomal Amphotericin B has been shown to decrease toxicity effects. Entrapping of Amphotericin B in liposomal formulation allows patients to receive higher doses for effective treatment while conventional Amphotericin B treatment is limited because of the toxicity of conventional Amphotericin B formulations. Lipid complexes have been found to have minimal

* Corresponding author at: Department of Chemistry, Kirti M. Doongursee College, Mumbai University, Kashinath Dhuru Road, Mumbai 400 028, India.
Tel.: +91 9819930539.
E-mail address: niranjan.deshpande@cipla.com (N.M. Deshpande).

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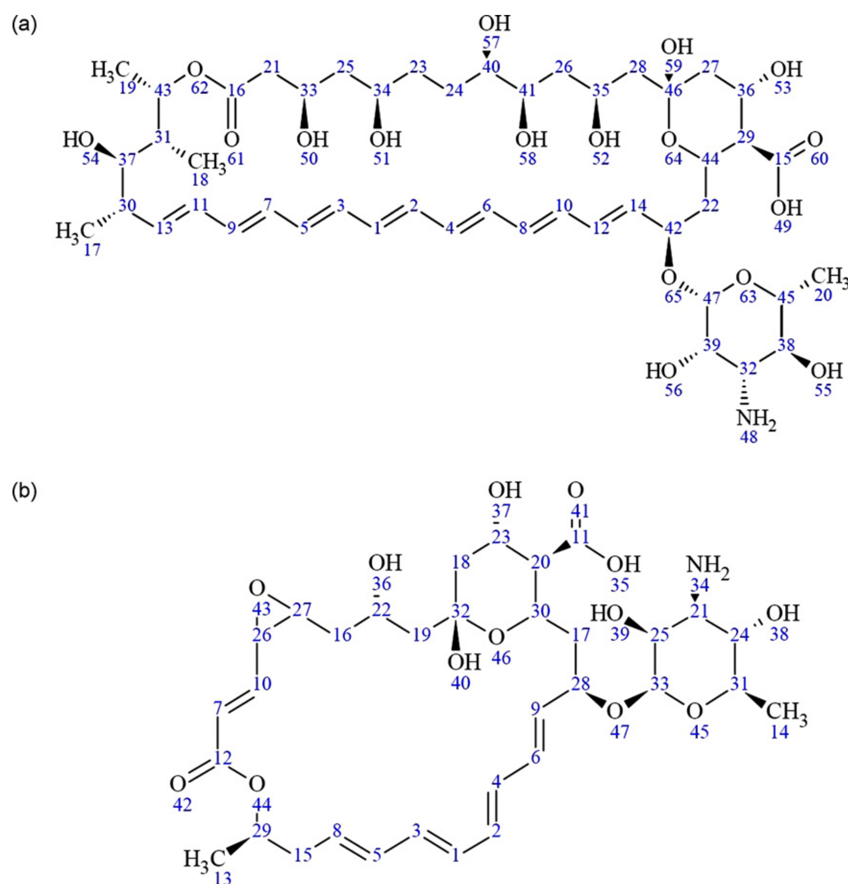

Fig. 1. Structures of Amphotericin and Natamycin. (a) Amphotericin B. (1*R*,3*S*,5*R*,6*R*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,19*E*,21*E*,23*E*,25*E*,27*E*,29*E*,31*E*,33*R*,35*S*,36*R*,37*S*)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid. (b) Natamycin. (1*R*,3*S*,5*R*,7*R*,8*E*,12*R*,14*E*,16*E*,18*E*,20*E*,22*R*,24*S*,25*R*,26*S*)-22-[(3-amino-3,6-dideoxy-D-mannopyranosyl)oxy]-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.$0^{5,7}$]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid.

interactions with mammalian cells, and thus are directed to the site of infections [2]. The amount of conventional Amphotericin B, i.e. free Amphotericin B (F-AMP) which is not entrapped in the liposomal preparation can increase the toxicity of the formulation and hence it is necessary to measure the amount of this free Amphotericin B along with liposomal Amphotericin B (L-AMP), i.e. amount of drug entrapped in the liposomal formulation. The separation between free Amphotericin B and liposomal Amphotericin B is challenging due to limited solubility of the drug and the stability of the liposome with respect to holding of drug in trapped state. The separation method should be designed in such a way that it should achieve the consistent extraction efficiencies and recoveries of free drug in human plasma over the linearity range chosen for pharmacokinetic studies. For determination of free Amphotericin B, this has to be achieved without disturbing or breaking the liposomes present in human plasma. High-pressure liquid chromatography (HPLC) methods were reported for the quantification of Amphotericin B in plasma or serum [6–20]. These methods quantified the total amount of Amphotericin B in circulation. Egger et al. [21] reported determination of Amphotericin B, liposomal Amphotericin B and Amphotericin B colloidal dispersion in plasma by HPLC with detection at 405 nm. They used solid phase extraction technique for determination of both F-AMP and L-AMP and reported recoveries were around 95% for lower concentrations but were only around 75% for higher concentrations. In addition, the linearity range was 0.5–5 μg/ml for L-AMP and 0.005–0.5 μg/ml for F-AMP. Since C_{max} for Amphotericin B was reported by Bekersky et al. [22] to be 22.9 ± 10 μg/ml after administration of liposomal Amphotericin B at therapeutic dose of 2 mg/kg, it was necessary to establish the linearity of method for assay of liposomal Amphotericin B at least up to 80 μg/ml (i.e. 3.5 times the C_{max} value). Considering that the free Amphotericin B present in plasma after dosing of liposomal Amphotericin B to human subjects, would be up to 15% with respect to concentration of liposomal Amphotericin B, the linearity of free Amphotericin B was also required to be established at least up to 12 μg/ml. The recovery by Egger's method was only around 75% at higher concentrations (2 μg/ml and 5 μg/ml). The recovery would drop further with concentration range beyond 5 μg/ml and it would lead to a non-linear nature of calibration curve. Hence, there was a need to develop a method with better and consistent recoveries over the entire range of calibration curve, i.e. from lower to higher concentration in calibration curve. Egger et al. [21] did not mentioned about the use of internal standard in the solid phase extraction procedure developed by them. Such a method would demand an extreme care to control all the processes and the variable recovery could contribute to inconsistent results. In addition to this, the HPLC methods suffered from potential interferences from endogenous materials in plasma or serum [23]. Liquid chromatography–mass spectroscopy (LC–MS–MS) has better selectivity than HPLC methods. The potential interference of bilirubin and other yellow components in plasma could be avoided by such a selective method [23]. The HPLC methods reported longer run times, typically more than 10 min. The selectivity and specificity required for determining Amphotericin B in plasma could be achieved by using LC–MS–MS technique with faster analysis time therefore, it was selected for the bioanalytical assay of Amphotericin B. The LC–MS–MS method developed by Lee et al. [23] used Symmetry C18 analytical column (150 mm × 4.6 mm, 5 μm) with

mobile phase flow rate of 0.5 ml/min. The runtime was 3.5–4.0 min. The molecular ions for Amphotericin B were set at m/z 924 and product ion at m/z 906. Similar method with modifications was used by Hong et al. [24] for the determination of free Amphotericin B from ultrafiltrate. However, Lee et al. [23] did not mentioned about separation of inherent impurities present in Amphotericin B by chromatography method selected by them. Amphotericin B showed inherent peaks of impurities. These impurities also showed molecular ions and product ions at m/z 924 and 906 respectively. Hence, these impurities possibly could be isomers Amphotericin B and showed response in mass spectra with same multiple reaction monitoring (MRM) transition as that of Amphotericin B. Therefore, there was a need to separate these probable isomeric impurities chromatographically from main Amphotericin B peak to achieve the specificity of assay method.

The aim of the present work was to develop a specific, fast, accurate and robust method to separate and quantify the two species F-AMP and L-AMP in human plasma after administration of Amphotericin B liposomal formulation intravenously. The method can be utilized for quantification of F-AMP and L-AMP in pharmacokinetic studies.

2. Experimental

2.1. Materials and reagents

Working standard (W.S.) of Amphotericin B used was prepared against pharmacopoeial reference standard of United States. Purity of W.S. was >99.0%. Natamycin pharmacopoeial reference standard of United States was used as an internal standard. AmBisome, a liposomal Amphotericin B injection was procured from Gilead Sciences Ltd., UK. HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Ammonium acetate was procured from Fluka Analytical, Sigma–Aldrich Chemie GmbH, Steinheim, Netherlands. Glacial acetic acid and chloroform were procured from Merck Specialities, Mumbai, India. Dimethyl sulphoxide A.R. grade was procured from Labscan Analytical Sciences, Patumwan, Bangkok. Purified water was generated from TKA water purification system. Oasis HLB 1 cm^3, 30 mg solid phase extraction cartridges were procured from Waters (India) Pvt. Ltd. Blank human plasma was collected with sodium heparin anticoagulant from healthy and drug free volunteers and stored at −70 °C.

2.2. Instrumentation and LC–MS conditions

The liquid chromatography system (Agilent 1200 series, USA) composed of Quaternary pump G1311A, Thermostated auto sampler HiP-ALS G1367B and column oven G1316A. The column Chromolith Performance RP18e (100 mm × 4.6 mm) from Merck (India) was used. The mobile phase eluent 'A' composed of 48 vol. of 5 mM ammonium acetate buffer, pH adjusted to 6.0 with glacial acetic acid, mixed with 20 vol. of acetonitrile and 32 vol. of methanol. The mobile phase eluent 'B; composed of 25 vol. of 5 mM ammonium acetate buffer, pH adjusted to 6.0 with glacial acetic acid, mixed with 5 vol. of acetonitrile and 70 vol. of methanol. The gradient program was 100% flow of eluent 'A' till 2.5 min and 100% flow of eluent 'B' from 2.5 min to 5.5 min. One-minute flow with eluent 'A' was set as equilibration period before next injection. Flow rate of 1.8 ml/min was used throughout the run. Total run time was of 6.5 min.

An API 4000 LC–MS/MS triple quadrupole mass spectrometer equipped with a Turbo Ion Spray™ ionization source (Applied Biosystems/MDS Sciex, Toronto, Canada) was used for detection of LC eluents. The operating parameters of the ionization source, including analyte dependant parameters and source dependant parameters were optimized to obtain optimum performance of the mass spectrometer for the analysis. The analysis was performed in MRM and positive ionization mode. The mass transitions 924.5 → 906.6 for Amphotericin B and 666.2 → 503.4 for Natamycin (IS) were used (Fig. 2a and b). The source dependant parameters such as curtain gas, collision gas, nebuliser gas (gas 1), heater gas (gas 2), ion spray voltage and the temperature of the heater gas were optimized to values 20, 10, 50, 30, 5500 V and 450 °C respectively. The analyte dependant parameters set for Amphotericin B and Natamycin were declustering potential (DP): 76 V and 61 V; collision energy: 19 V and 17 V respectively. Entrance potential was set at 10 V for both Amphotericin B and Natamycin. Dwell time was kept at 0.5 s. Data were acquired and processed using Analyst version 1.4.1 software.

2.3. Preparation of standards and QC samples

Stock solutions were prepared in two sets. One set was referred to as Part 'A' and prepared for determination of amount of free Amphotericin B (F-AMP). Another set was referred to as Part 'B' and prepared for determination of liposomal Amphotericin B (L-AMP).

Part A: Stock solutions for Part 'A' were prepared by dissolving and diluting volumetrically W.S. of Amphotericin B in methanol. Initial stock solution was prepared in duplicate and had concentration of 200 μg/ml. The calibration curve stocks for free Amphotericin B were prepared by serially diluting stock solution with methanol. Each calibration curve stock was spiked in previously screened blank plasma individually, resulting in calibration curve standards having plasma concentrations of 0.25, 0.75, 1.50, 3.0, 5.0, 7.5, 10.0 and 15.0 μg/ml. Plasma concentrations of 0.25, 0.75, 5.0 and 10.0 μg/ml were used as lowest level of quantification (LLOQ), lower level of quality control (LQC), middle level of quality control (MQC) and higher level of quality control (HQC) respectively (collectively defined as F-AMP QCs).

Part B: Stock solutions for part 'B' were prepared by reconstituting the lyophilized cake of Amphotericin B liposomal formulation for injection with HPLC grade water. Reconstituted solution was then diluted serially with HPLC grade water to prepare the calibration curve stocks for liposomal Amphotericin B. Each calibration curve stock was spiked in previously screened blank plasma individually, resulting in calibration curve standards having concentrations of 1.0, 3.0, 10.0, 20.0, 30.0, 60.0, 80.0, and 100.0 μg/ml. Plasma concentrations of 1.0, 3.0, 30.0 and 80.0 μg/ml were used as LLOQ, LQC, MQC and HQC respectively (collectively defined as L-AMP QCs). W.S. of Amphotericin B was used to prepare stock solutions of same concentrations, as that of L-AMP QCs, by diluting in 1:1 mixture of DMSO and methanol. These stocks of W.S. were prepared for checking the recovery of L-AMP after extraction from plasma.

Internal standard stock solution was prepared by dissolving and diluting volumetrically, reference standard of Natamycin in methanol. The concentration of internal standard stock solution used for assessing liposomal Amphotericin B was 300.0 μg/ml, whereas for free Amphotericin B the concentration was 60.0 μg/ml.

All these stock solutions were prepared in volumetric flasks and were stored at 2–8 °C in refrigerator. Stock solutions were brought to room temperature before use. Plasma calibration curve standards and Quality control samples were stored at −70 °C in deep freezer.

2.4. Preparation of plasma samples and extraction procedures

All frozen subject samples, calibration standards and quality control samples were thawed at room temperature prior to analysis. The samples were adequately vortexed. An aliquot of 250 μL plasma was mixed with 25 μL internal standard Natamycin (stock

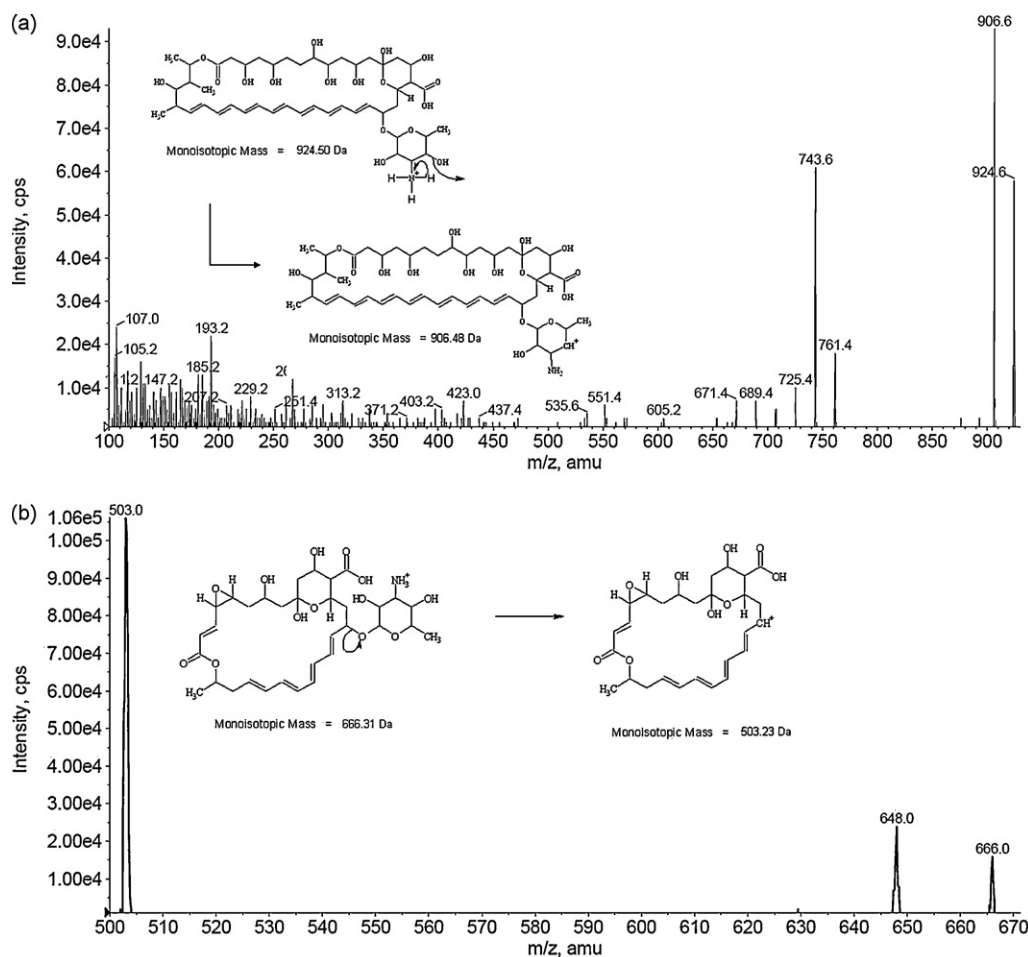

Fig. 2. MS fragments of Amphotericin B and Natamycin (internal standard). (a) MS fragmentation spectra of Amphotericin B. (b) MS fragmentation spectra of Natamycin.

concentration 60 μg/ml) and 500 μL of 0.1% aqueous ammonia solution. The mixture was vortexed.

Part A: The Oasis HLB SPE cartridge was preconditioned with 1.0 ml methanol followed by 1.0 ml of water. The plasma sample mixture was loaded onto the SPE cartridge and eluate was collected in fresh test tube. Further, 250 μL of 0.1% aqueous ammonia solution was added to the test tube, vortexed and it was again loaded onto the same SPE cartridge. The cartridge was washed with 250 μL water to wash off remaining plasma and liposomal Amphotericin B remaining on the cartridge. These fractions were collected in the same test tube and kept as fraction "B" for the analysis of liposomal Amphotericin B. The SPE cartridge was washed with 1.0 ml of water and 1.0 ml of 5% aqueous methanol (to remove heparin plasma and maybe other interfering substances). The free Amphotericin B, which was retained on SPE cartridge, was then eluted with 2.0 ml methanol and referred to as Fraction "A". Analysis of fraction "A" by LC–MS–MS gave the amount of free Amphotericin B in plasma (F-AMP).

Part B: Fraction "B" was spiked with 25 μL of Natamycin (stock concentration 300 μg/ml) and was heated on a water bath at 40 °C for 30 min. After cooling 25 μL chloroform and 1.75 ml of 2% DMSO in acetonitrile were added and was sonicated (to break liposomes and to solubilize Amphotericin B in acetonitrile–DMSO mixture). Sample was vortexed and then centrifuged at 4660 × *g*. 200 μL of the clear layer was diluted to 1.2 ml with methanol (to make necessary concentration for LC–MS–MS injection) and then was injected on LC–MS–MS. This diluted fraction was referred to as fraction "C". The analysis of fraction "C" gave amount of liposomal Amphotericin B in plasma (L-AMP).

2.5. Method validation

The method was validated for specificity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, stability and dilution integrity according to US FDA guidelines [25]. Additional parameter validated was separation efficiency between free and liposomal Amphotericin B.

Selectivity and specificity were performed using 8 different sources of blank plasma with sodium heparin added as anticoagulant. Six sources of normal blank plasma, one source each of haemolysed and lipemic blank plasma were selected. They were processed by extraction protocol for collection of both fractions "A" and "C" and their responses were assessed at retention time of Amphotericin B and internal standard. Six LLOQ samples (three each of F-AMP QCs collected at fraction "A" and L-AMP QCs collected at fraction "C") were prepared from chromatographically screened blank plasma.

Intra-batch and inter-batch precision and accuracy of the method were determined on 4 different days at 4 different concentration levels of LLOQ, LOQ, MQC and HQC ($n = 7$) for each type of quality control sample F-AMP QCs and L-AMP QCs. The precision was calculated in terms of coefficient of variation (%CV). Accuracy was calculated in terms of the degree of closeness of back calculated concentration value, calculated from calibration curve, to nominal concentration value. For accuracy study deviation from nominal value should not be more than 15%, i.e. accuracy should be within 85–115%.

Recovery of extraction of free Amphotericin B from human plasma with SPE procedure was calculated using 3 levels of F-AMP

QCs (LQC, MQC and HQC, $n = 7$). The areas of fraction “A” collected for three F-AMP QC levels were compared against unextracted samples of similar concentration spiked in methanol. Recovery of liposomal Amphotericin B was calculated using 3 levels of L-AMP QCs (LQC, MQC and HQC, $n = 7$). The areas of fraction “C” collected for three L-AMP QC levels were compared against unextracted samples of similar concentration prepared by using stock solutions of Amphotericin B W.S. spiked in diluting solution.

Matrix effect experiment was performed by post column infusion and post extraction addition to evaluate the interference, if any, from endogenous materials of human plasma, at retention times of analyte and internal standard. A standard solution prepared in methanol at F-AMP MQC level was infused post column using T-connector into mass spectrometer at 10 μL/min infusion rate. Six different sources of blank human plasma were extracted to collect both the fractions “A” and “C”. 5 μL of these fractions was injected into the LC–MS–MS system. Acquired LC–MS–MS chromatograms were observed for a baseline enhancement or suppression at the retention times of analyte and internal standard. To study the matrix effect on quantification of analyte, six different sources of blank human plasma were processed and fraction “A” & “C” were collected. F-AMP LQC and HQC standard stock solutions were spiked in triplicate in fraction “A” and L-AMP LQC and HQC standard stock solutions of Amphotericin B W.S. were spiked in triplicate in fraction “C”. The responses of LQC and HQC spiked in processed blank plasma samples were compared with respective QC standards spiked in methanol or diluting solution. The recoveries were calculated by comparing relative responses of QC standards spiked in blank plasma to QC standards spiked in methanol or diluting solution.

Stability experiments were performed to evaluate analyte stability in stock solutions and in plasma samples under different conditions. Stability of Amphotericin B stock solutions for free Amphotericin B as well as stability of internal standard stock solutions were determined by comparing area response of stability stock solution samples with area response of freshly prepared stock solutions.

Stability of stock solutions for liposomal Amphotericin B was determined by comparing the recovery of liposomal Amphotericin B stability stock solution samples at L-AMP QCs (LQC, MQC and HQC, $n = 3$) after collection of fraction “C” with recovery of freshly prepared liposomal Amphotericin B stock solutions. Results for stability liposomal stock solutions were monitored for not more than 15% drop in recovery of liposomal Amphotericin B in stability samples.

Bench top stability, extracted sample stability (post preparative), freeze–thaw stability and long-term stability were performed at LQC and HQC levels of F-AMP QCs and L-AMP QCs using seven replicates at each level. Results for stability experiments were monitored for ±15% change in stability.

The dilution integrity experiment was carried out at 2.0 times higher concentration of highest level of calibration curve (ULOQ), i.e. 30.0 μg/ml for free Amphotericin B and 200.0 μg/ml for liposomal Amphotericin B. This dilution integrity stock in plasma was then diluted three times and five times with blank human plasma. Seven replicates of such 1/3rd and 1/5th concentrations were prepared and processed along with freshly prepared calibration curve. Their mean back calculated concentrations were calculated when processed against freshly prepared calibration curve. The deviation of mean back calculated concentration values should be in the range of 85–115% with respect to their nominal values after application of appropriate dilution factor of 3 and 5 respectively.

Separation efficiency experiment was carried out to observe the separation between free and liposomal Amphotericin B. In 250 μL human plasma each QC level of free Amphotericin B (F-AMP LQC, MQC and HQC) was spiked together with each QC level of Liposomal Amphotericin B (L-AMP LQC, MQC and HQC). Spiking was done in such a way that each 250 μL human plasma contained one F-AMP QC level and one L-AMP QC level. The spiked QCs were then accessed for determination of free and liposomal Amphotericin B. QCs were processed along with fresh calibration curves of free and liposomal Amphotericin B. Separation efficiency was determined in terms of accuracy of free and liposomal QCs after extraction of spiked samples. Accuracies were determined for each QC level after extraction by comparing nominal concentration and back calculated concentration determined after extraction. The deviation of back calculated concentration from nominal value should not be more than 15%, i.e. accuracy should be within 85–115%.

3. Results and discussion

3.1. Method development

Mass spectroscopy (MS) parameters were optimized for achieving selectivity and sensitivity in positive ion mode. Natamycin which is structurally similar polyene was selected as internal standard. Both the analyte and internal standard have ability to accept proton and generate $[M+H]^+$ ions. Protonated parent ions for Amphotericin B and Natamycin were observed at m/z 924.5 and 666.2 respectively. The analyte and internal standard were then fragmented in collision cell by use of nitrogen as collision gas. The fragments were selected at m/z 906.6 and 503.4 as most prominent and stable fragments for Amphotericin B and internal standard respectively (Fig. 2a and b). Nebulizer and evaporator gases were optimized with flow injection analysis (FIA) mode. Selection of internal standard is important as the quantification is made by analyte to internal standard area ratio. Thus, internal standard must have similar physicochemical properties as that of analyte. SPE recoveries and protein precipitation recoveries were consistent and were 100% for selected internal standard Natamycin.

For optimization of chromatographic conditions, mobile phase composition was varied using buffers with different strengths and pH. Amphotericin B is a hydrophobic compound and it was observed that pH and buffer strengths did not significantly affect the chromatographic retention and MS response of Amphotericin B. Amphotericin B has two pK_a values, 8.12 which is basic pK_a due to amino group at position 48 and 3.72 which is acidic pK_a due to one acidic group at position 15 (Fig. 1). Hence, pH 6.0 was selected which is slightly acidic and suitable for most reverse phase HPLC columns and pH 6 is also in the range of ±2 of both pK_a values resulting in keeping Amphotericin B in one ionic state for MS analysis. Ammonium acetate buffer was selected with 5 mM strength to avoid overloading of ionization source with buffer. Amphotericin B has a long aliphatic polyene chain. A very broad and asymmetric peak shape was observed with 120 Å pore size HPLC columns. Various columns, which were having pore size up to 200 Å were tried to improve peak shape. In addition, Amphotericin B showed inherent peaks of impurities (labeled as P and Q, see Fig. 3a). Amphotericin B and the impurities P and Q showed parent and daughter ions with m/z 924 and 906 respectively (see Fig. 3 b, c and d). Hence, these impurities possibly could be isomers Amphotericin B and showed response in mass spectra with same MRM transition as that of Amphotericin B. Therefore, there was a need to separate these probable isomeric impurities P and Q chromatographically from main Amphotericin B. In order to achieve separation between internal standard, probable isomeric impurities P and Q and Amphotericin B, a gradient elution program was set which was having high concentration of buffer initially. Amphotericin B being non-polar molecule retained on column at this stage. The organic solvent concentration was increased linearly to elute Amphotericin B and separate impurities. This separation resulted in longer run time up to 9 min. To reduce run time, higher flow rates need to be selected. This

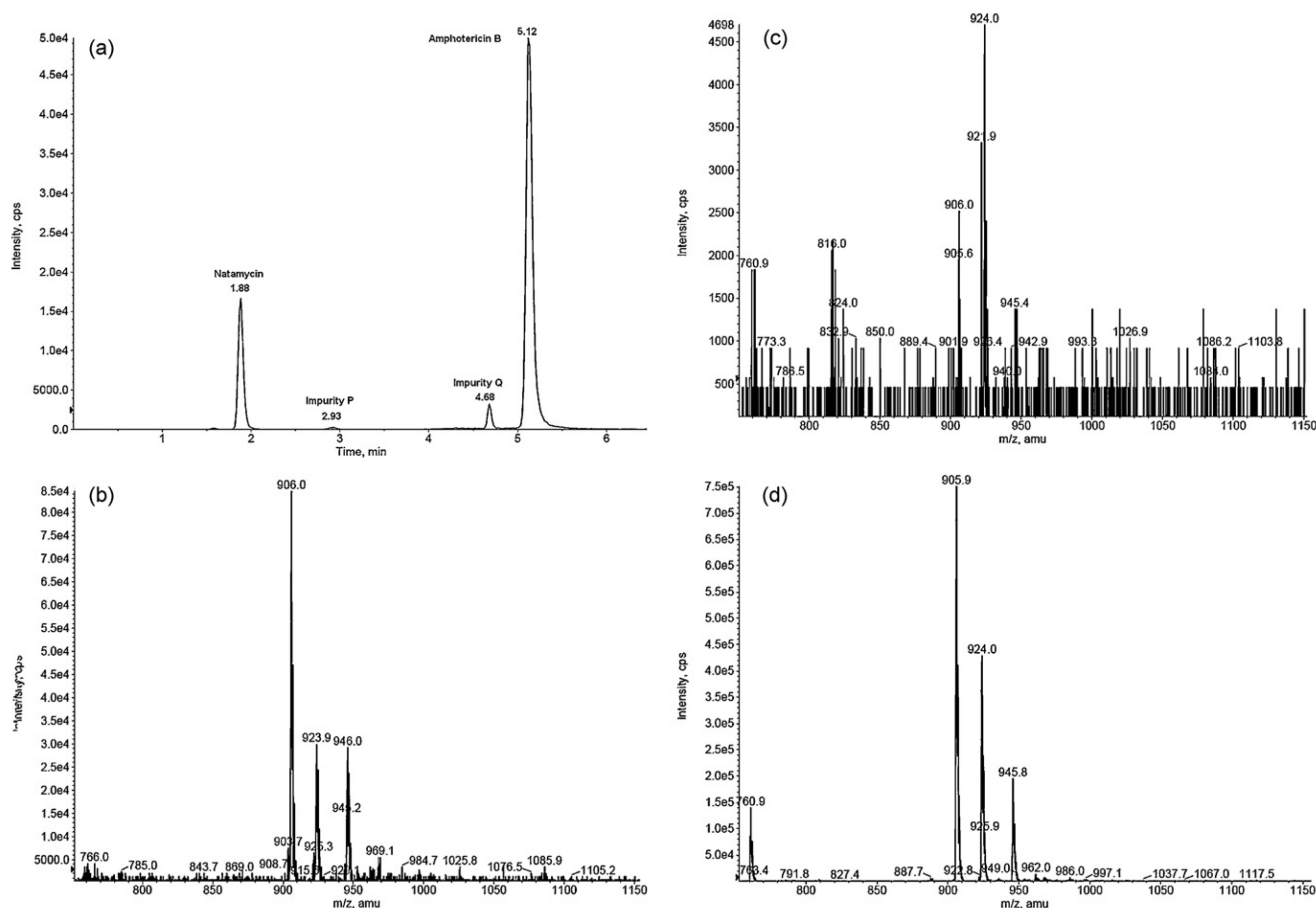

Fig. 3. (a) LC–MS–MS MRM chromatogram showing separation between Natamycin (at about 1.9 min), Amphotericin B (at about 5.1 min) and impurities P and Q having response at same MRM transitions (at about 2.9 min and 4.7 min). (b–d) Mass spectra of Amphotericin B and impurities P, Q, showing same parent and daughter ions at m/z 924 and 906 respectively. (peak at m/z 946 is due to sodium adduct of parent ion 924 amu). (a) LC–MS–MS MRM chromatogram. (b) Mass spectra of Amphotericin B at about 5.1 min. (c) Mass spectra of impurity P at about 2.9 min. (d) Mass spectra of impurity Q at about 4.7 min.

requires an analytical column which can be run with flow rates up to 2 ml/min with minimum backpressure and which can give proper symmetric peak shapes of analyte and internal standard. Chromolith Performance RP 18e column having 4.6 mm internal diameter and 10 cm length was selected which showed good peak shape and an asymmetry factor less than 2.0 for Amphotericin B and internal standard. Flow rate of 1.8 ml/min was selected to shorten the run time. Resultant run time was 6.5 min with proper separation between internal standard, Amphotericin B and its impurities (see Fig. 3a). The retention time for internal standard was at about 1.9 min and for Amphotericin B was at about 5.1 min.

The challenge in developing sample extraction method was to separate precisely and accurately free Amphotericin B from liposomal Amphotericin B. Amphotericin B cannot exist as free entity in plasma as it binds with plasma proteins [21,26]. To achieve the separation between free and liposomal Amphotericin B, solid phase extraction with Oasis HLB cartridges was performed. The Liposomal Amphotericin B was not retained on the SPE cartridge and eluted out. Free Amphotericin B bound to plasma protein had to be retained on SPE cartridge with the help of addition of buffers or specific reagents, which can help in holding free Amphotericin B on the SPE cartridge and enabling plasma to be washed off from cartridge. Selection of such a reagent or buffer was very critical, as it should be efficient enough for retaining Amphotericin B on SPE cartridge over the entire linearity concentration range (for F-AMP from 0.25 μg/ml to 15 μg/ml) and it should not break or adversely affect the liposomes present in the sample. This was achieved by using 500 μL of 0.1% aqueous ammonia solution. The strength of aqueous ammonia solution was limited to 0.1% in such a way that it should not break liposomes present in plasma samples and the separation efficiency can be proven. It was observed that the recoveries were not good at higher concentrations of free Amphotericin B by solid phase extraction. When the eluate was reloaded on the same SPE cartridge along with additional amount of 250 μL of 0.1% aqueous ammonia solution, there was sufficient interaction of Amphotericin B with SPE bed and hence all the free Amphotericin B was retained on the SPE cartridge. This improved the recovery of free Amphotericin B considerably. It was necessary to have consistent and higher recoveries of free Amphotericin B so that the un-retained free Amphotericin B on SPE cartridges would not interfere in the quantification of liposomal Amphotericin B. With the use of 0.1% aqueous ammonia solution and reloading method recoveries of free Amphotericin B were linear and consistent over entire linearity range (recovery >90% from F-AMP LLOQ (0.25 μg/ml) to F-AMP ULOQ (15.0 μg/ml) level). There was no adverse effect due to breaking of liposomes on accuracy of free Amphotericin B in plasma standards spiked together with F-AMP and L-AMP stocks; hence, the separation efficiency was established by this method. The fraction "B" was collected after reloading on SPE cartridge and washing the cartridge with water. This fraction "B" contains liposomal Amphotericin B along with plasma constituents. This fraction was warmed in a water bath at 40 °C to break the liposomes. Lipids of liposomes have solubility in chloroform and DMSO hence 25 μL chloroform and 1.75 ml of 2% DMSO in acetonitrile was added. The volume of DMSO was selected in such a way that it should be sufficient enough to dissolve Amphotericin B extracted from liposome

as well as it should have least interference in MS ionization of analyte and internal standard. The mixture was subjected to 30-min sonication for effective breaking of liposomes. Amphotericin B being a lypophilic moiety has a tendency to stick to broken lipids of liposomes. Sonication helped to dissolve Amphotericin B in DMSO and acetonitrile. Extraction recoveries for this fraction B were also consistent over entire linearity range of liposomal Amphotericin B (recovery >85% from L-AMP LLOQ (1.0 μg/ml) to L-AMP ULOQ (100.0 μg/ml) levels).

3.2. Selectivity and specificity

Chromatograms in Fig. 4 demonstrate the selectivity, specificity and sensitivity of the method. Retention times of 1.9 min and 5.2 min for internal standard Natamycin and Amphotericin B respectively were observed. There was no significant interference from blank plasma extracted from eight different sources either by SPE procedure or by protein precipitation procedure at these retention times.

3.3. Linearity

The four calibration curves were linear from 0.25 μg/ml to 15.0 μg/ml for F-AMP with correlation coefficient $r \geq 0.9986$ and from 1.0 μg/ml to 100.0 μg/ml for L-AMP with correlation coefficient $r \geq 0.9956$. The straight-line fit was made through the data points to give the linear equation $y = 0.174x + 0.0081$ for F-AMP and $y = 0.0329x + 0.0124$ for L-AMP, where y is peak area ratio of Amphotericin B to internal standard and x is concentration of Amphotericin B. The weighting factor $1/X^2$ was used to calculate correlation coefficient, slope and intercept. The standard deviation values for slope, intercept and correlation coefficient (r) were 0.019, 0.0043 and 0.0003 respectively for F-AMP and 0.003, 0.002 and 0.0017 respectively for L-AMP.

3.4. Intra-batch and inter-batch precision and accuracy

For inter-batch, four different analytical runs and for intra-batch, a single analytical run was assayed. Each run contains seven replicates of four concentration levels LLOQ, LOQ, MQC and HQC of free Amphotericin B and liposomal Amphotericin B each. Intra-batch and inter-batch precision (%CV) values were from 1.42% to 6.31% for free Amphotericin B and from 3.38% to 8.90% for liposomal Amphotericin B. The intra-batch and inter-batch accuracy for developed method was from 96.9% to 104.8% for free Amphotericin B and from 92.04% to 105.8% for liposomal Amphotericin B with respect to their nominal concentrations as shown in Table 1.

3.5. Recovery

The recoveries found at F-AMP LQC, MQC and HQC levels were 91.2%, 98.6% and 97.7% respectively. The precision (%CV) among three F-AMP QC levels found was 2.77%, 3.15% and 4.52% respectively. The global average recovery of all twenty-one F-QCs was 95.8%. The recovery of internal standard was 103.5% with a %CV 3.75 ($n = 21$). The recoveries found at L-AMP LQC, MQC and HQC levels were 95.4%, 93.4% and 87.3% respectively. The precision (%CV) among three L-AMP QC levels found was 3.53%, 9.89% and 3.76% respectively. The global average recovery of all twenty-one L-AMP QCs was 92.1%. The recovery of internal standard was 103.3% with a %CV 3.68 ($n = 21$).

3.6. Matrix effect

No major suppression/enhancement was observed at the retention times of Amphotericin B and internal standard due to matrix effect (as shown in Fig. 5 by post column infusion). Further, the effect of matrix in quantification of Amphotericin B was studied by post extraction addition method. The recovery of QCs added in blank plasma after extraction was consistent for all selected plasma lots. Recovery was from 98.3% to 102.9% for free Amphotericin B and from 103.8% to 112.0% for liposomal Amphotericin B. The %CV was 1.68% and 1.22% for free Amphotericin B at F-AMP LQC and F-AMP HQC levels respectively. The %CV was 2.09% and 1.50% for liposomal Amphotericin B at L-AMP LQC and L-AMP HQC levels respectively. Hence, matrix effect was not observed in the quantification of free and liposomal Amphotericin B.

3.7. Stability

Stock solutions of F-AMP and internal standard were stable at room temperature and at 2–8 °C for seven days. Stock solution stability of F-AMP was 99.3% and 103.4% at room temperature and 2–8 °C respectively. Stock solution stability of L-AMP stocks cannot be determined by direct comparison of responses of stability stock solutions and freshly prepared stock solutions as Amphotericin B is trapped in liposomal cavity, hence, the stability was determined by observing change in recovery of stability stock solutions after extraction with respect to recovery of freshly prepared stock solutions. The stocks of liposomal Amphotericin B were stable after seven days storing at 2–8 °C and stock solution stability of liposomal Amphotericin B was 107.2%.

Stability observations of Amphotericin B are shown in Table 2. F-AMP and L-AMP in human plasma were stable at least for 4.0 h at room temperature. The processed samples of F-AMP and L-AMP were stable for 48 h at 2–8 °C. Plasma samples of F-AMP and L-AMP spiked in human plasma were stable at −70 °C for 30 days. The QCs of F-AMP spiked in human plasma were stable for three freeze–thaw cycles at −70 °C. However, it was observed that the liposomal Amphotericin B is stable only for one freeze and thaw cycle. The stability at L-AMP LQC level goes on decreasing after each successive freeze–thaw cycle. This can be explained as the liposomes tends to break during temperature alterations of freeze–thaw cycles and the Amphotericin B released from liposomes retained on SPE cartridge as a fraction of free Amphotericin B. This resulted in decrease in concentration of liposomal Amphotericin B at fraction "B" collected after SPE procedure.

3.8. Dilution integrity

The experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ) which may be encountered during real subject samples analysis. Such samples are supposed to be diluted with blank human plasma to achieve appropriate concentrations within calibration curve range. The experiment was carried out at 2.0 times higher concentration of ULOQ and diluted with blank human plasma by dilution factors 3 and 5. Dilution factors 3 and 5 were selected to measure dilution integrity of real subject samples having concentrations at least three times or maximum five times more than the ULOQ level. The mean back calculated concentrations of 1/3rd and 1/5th diluted samples for F-AMP were 99.99% and 99.45% respectively to their nominal values. The coefficients of variance (%CV) were 3.18% and 2.15%. The mean back calculated concentrations of 1/3rd and 1/5th dilution samples for L-AMP were 102.8% and 105.50% respectively to their nominal values. The coefficients of variance (%CV) were 2.05% and 1.87%.

3.9. Separation efficiency

Separation efficiency between free and liposomal Amphotericin B was validated to ensure free and liposomal states of Amphotericin

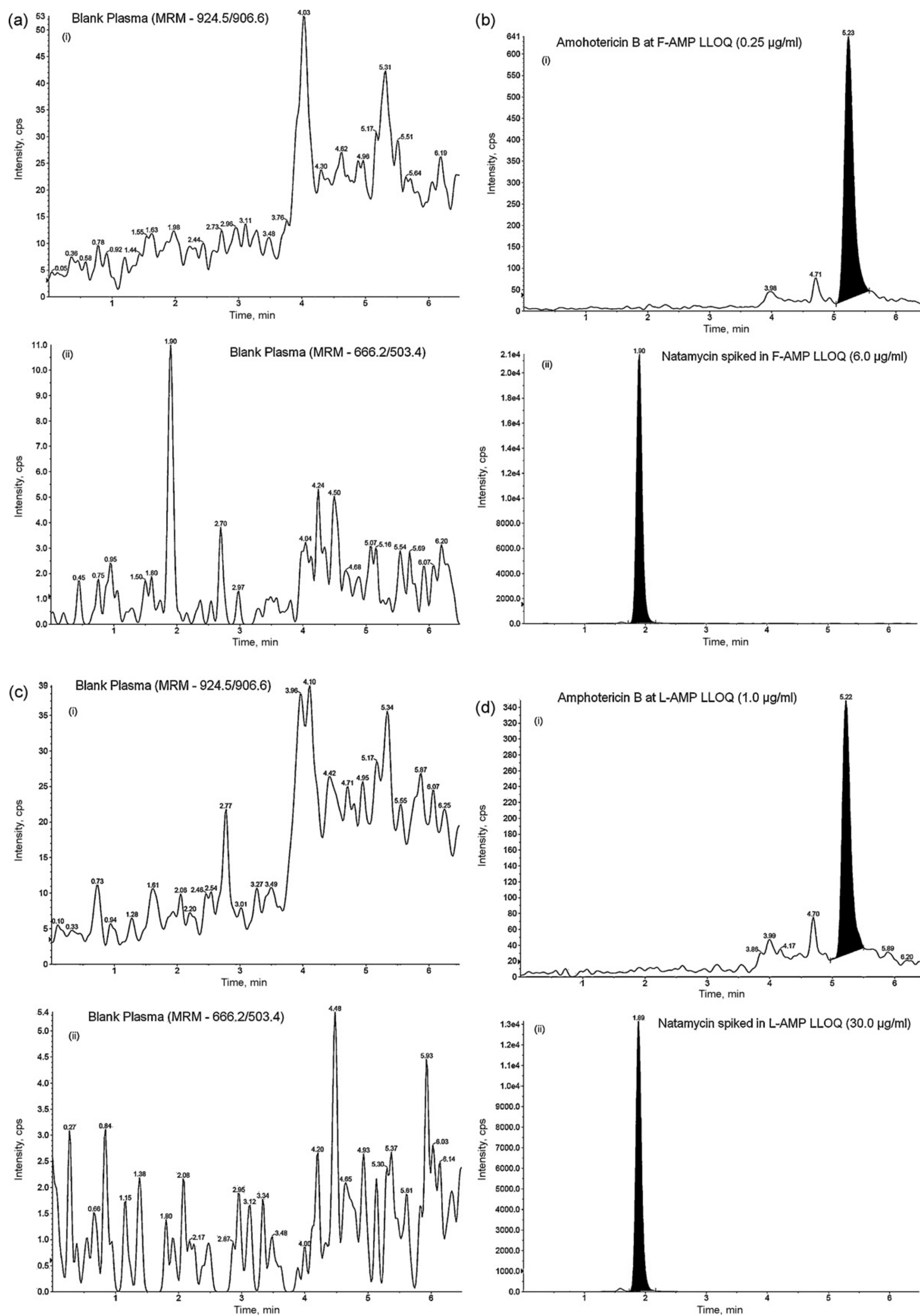

Fig. 4. MRM chromatograms of (a) Blank plasma at MRM 924.5/906.6 of Amphotericin B and 666.2/503.4 of Natamycin, extracted as fraction 'A', (b) plasma sample spiked with Amphotericin B at F-AMP LLOQ (0.25 μg/ml) and Natamycin (6.0 μg/ml) and extracted as fraction 'A' for free Amphotericin B, (c) Blank plasma at MRM 924.5/906.6 of Amphotericin B and 666.2/503.4 of Natamycin extracted as fraction 'C', (d) plasma sample spiked with Amphotericin B at L-AMP LLOQ (1.0 μg/ml) and Natamycin (30.0 μg/ml) and extracted as fraction 'C' for liposomal Amphotericin B.

Table 1
Intra-batch and inter-batch precision and accuracy of free and liposomal Amphotericin B.

Level	Conc. added (μg/ml) – (nominal conc.)	Intra-batch				Inter-batch			
		n	Mean conc. found (μg/ml)[a]	Accuracy[b]	%CV[c]	n	Mean conc. found (μg/ml)[d]	Accuracy[b]	%CV[c]
F-LLOQ	0.258	7	0.267	103.65	3.35	28	0.257	99.74	3.63
F-LQC	0.773	7	0.772	99.91	2.98	28	0.749	96.90	2.67
F-MQC	5.150	7	5.367	104.21	3.04	28	5.259	102.12	1.42
F-HQC	10.300	7	10.794	104.80	6.31	28	10.547	102.40	2.46
L-LLOQ	1.00	7	1.02	101.57	8.31	28	1.06	105.80	4.04
L-LQC	3.00	7	2.91	97.03	4.77	28	2.76	92.04	4.48
L-MQC	30.01	7	29.77	99.22	8.90	28	30.52	101.69	4.60
L-HQC	80.02	7	78.85	98.53	3.38	28	82.62	103.24	5.14

Single analytical run was assayed for intra-batch and four analytical runs were assayed for inter-batch precision and accuracy. Each analytical run was composed of seven replicates of four QC levels.

[a] Mean of seven replicates observations at each run.
[b] Degree of closeness of back calculated value calculated from calibration curve to nominal concentration value monitored for deviation 100 ± 15%.
[c] Coefficient of variance.
[d] Mean of twenty-eight replicates observations over four different analytical runs.

B were effectively separated from plasma sample by the method and accurately quantified without having any interference in each other's extraction process. The results of separation efficiency are tabulated in Tables 3a and 3b. The accuracy values of F-AMP were from 85% to 107% with coefficient of variance less than 7% after separation and extraction. The accuracy values of L-AMP were from 103.8% to 112.9% with coefficient of variance less than 12.5%. The separation efficiency between liposomal and free Amphotericin B has been achieved as the accuracy of free and liposomal Amphotericin B was within acceptance criteria of 85–115% in all the cases except one. The accuracy of L-AMP was 153.5% in the sample, where F-AMP HQC level (10.1 μg/ml) was spiked in plasma along with L-AMP LLOQ level (0.996 μg/ml) of Amphotericin B, where the amount of free Amphotericin B in spiked plasma was much more than the liposomal Amphotericin B. This is inconsistent with real-time scenario, as free Amphotericin B content cannot exceed liposomal Amphotericin B content in human plasma after administration of therapeutic dose of liposomal Amphotericin B injection.

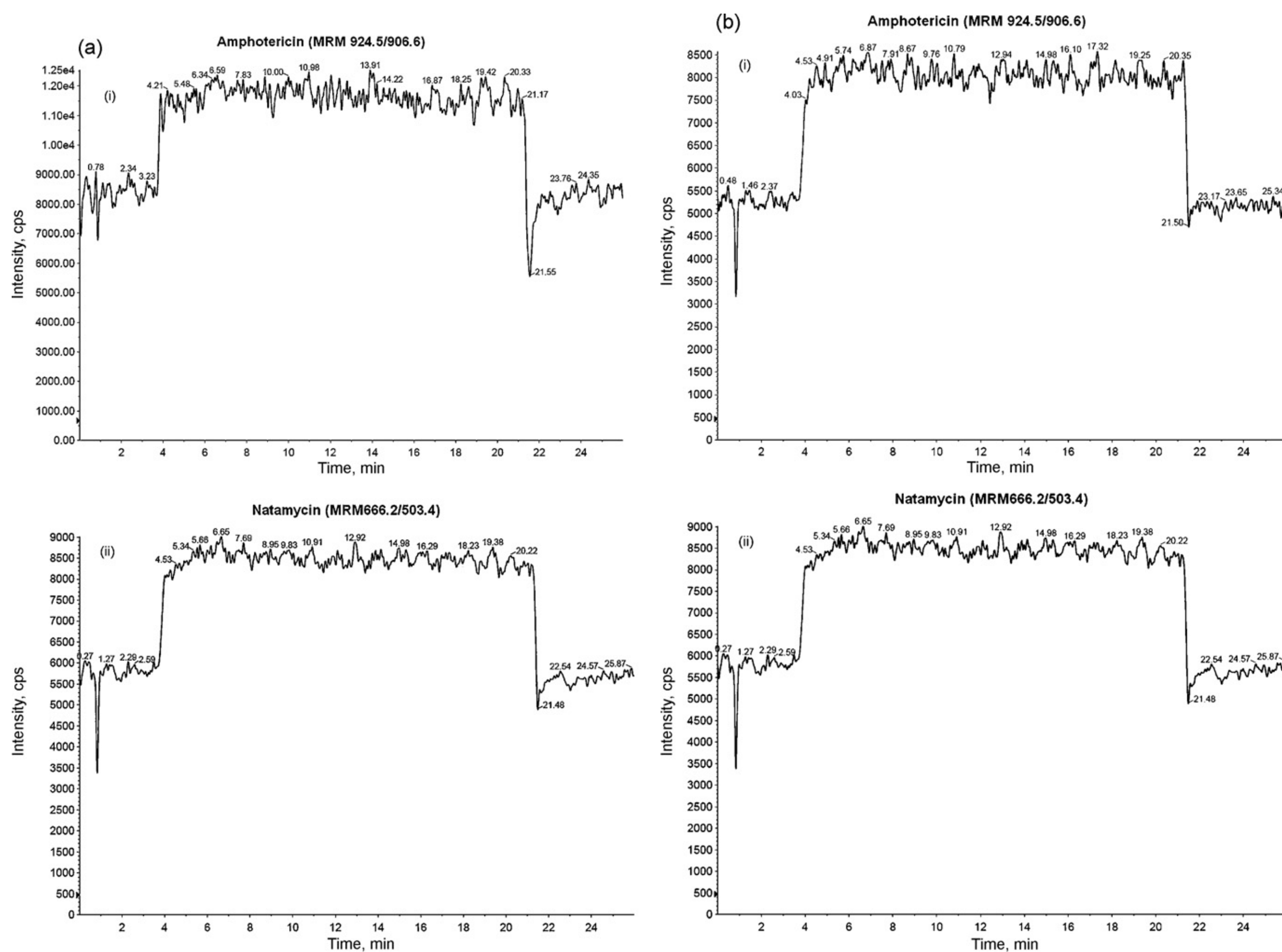

Fig. 5. Post column infusion MRM LC–MS–MS chromatograms of Amphotericin 924.5/906.6 and Natamycin 666.2/503.4 for (a) free Amphotericin extracted by SPE and (b) liposomal Amphotericin extracted as fraction "C" by protein precipitation.

Table 2
Stability results of Amphotericin B.

Stability condition	Storage condition	Level	Free Amphotericin B				
			A[a]	%CV[b]	B[c]	%CV[b]	% Stability[d]
Bench top	Room temperature–4 h	F-AMP LQC	0.74	1.74	0.75	2.96	100.61
		F-AMP HQC	10.61	4.03	10.36	3.34	97.68
Post preparative	After 48 h at 2–8 °C	F-AMP LQC	0.72	3.07	0.75	2.31	103.69
		F-AMP HQC	10.61	4.08	10.96	5.73	103.33
Freeze and thaw	After 3rd cycle at −70 °C	F-AMP LQC	0.764	2.54	0.760	3.58	99.48
		F-AMP HQC	10.66	3.76	10.00	4.92	93.80
Long-term stability	30 days at −70 °C	F-AMP LQC	0.77	2.98	0.78	2.55	100.37
		F-AMP HQC	10.79	6.31	9.81	4.72	90.92

Stability condition	Storage condition	Level	Liposomal Amphotericin B				
			A[a]	%CV[b]	B[c]	%CV[b]	% Stability[d]
Bench top	Room temperature–6 h	L-AMP LQC	2.75	1.72	2.88	3.05	104.72
		L-AMP HQC	82.37	4.85	86.27	5.16	104.73
Post preparative	After 48 h at 2–8 °C	L-AMP LQC	3.159	2.95	3.161	3.28	100.09
		L-AMP HQC	76.37	6.80	70.96	10.40	92.91
Freeze and thaw	Only once freezing and thawing at −70 °C	L-AMP LQC	2.92	2.35	2.68	3.02	91.86
		L-AMP HQC	82.76	2.56	79.80	3.77	96.43
Long-term stability	30 days at −70 °C	L-AMP LQC	2.92	4.78	2.61	3.07	89.37
		L-AMP HQC	79.33	3.38	78.89	4.42	99.43

[a] Mean comparison concentration in μg/ml, $n = 7$.
[b] Coefficient of variance.
[c] Mean stability concentration in μg/ml, $n = 7$.
[d] % mean change, acceptance criteria 100 ± 15%.

Hence, this result was not considered for conclusion derived for separation efficiency.

3.10. Application of method on human volunteers

The proposed method was successfully applied to bioavailability study of eight healthy human volunteers. Liposomal Amphotericin B injection having label claim of each vial containing Amphotericin B 50 mg encapsulated in liposomes were given to subjects under fed conditions. The therapeutic dose selected was 2 mg/kg body weight of subjects. The consent from subjects was obtained prior to study and approval of clinical protocol was done by independent ethics committee. Health check up for all subjects was done. Healthy willing volunteers from 18 to 45 years of age, having body-weight not less than 50 kg and not more than 70 kg were selected for study. Breath alcohol test and test for drugs of abuse were negative for selected subjects. Hepatitis A, B, C and antibodies to HIV were negative or non-reactive for selected subjects. Each subject received the formulation over a period of 2 h intravenous infusion. Each subject was given Acetaminophen ER (650 mg) and Diphenhydramine hydrochloride (50 mg) tablets 30 min prior to infusion of Amphotericin B with 240 ml of water. Concomitant medication of Acetaminophen ER and Diphenhydramine hydrochloride tablets were given to reduce possible side effects such as chills or fever and infusion reactions. The blood samples were collected in 24 h time intervals. The time intervals were pre-dose, and after 0.5–144.0 h post-dose. Blood samples were centrifuged at 1500–2000 × *g* at 10 ± 2 °C for 10 min. Sodium heparin was used as anticoagulant and plasma samples were stored at −70 °C until use. Depending on the validation results of freeze–thaw stability, precaution was taken for handling of study samples, that it should not undergo any freeze–thaw cycle apart from that of analysis. As the plasma samples contain the two additional drugs, Acetaminophen and Diphenhydramine hydrochloride, the validation was performed to check the specificity of the method for determining Amphotericin B in presence of these two drugs. The parameters assessed

Table 3a
Separation efficiency—accuracy of F-AMP after separation.

S. no.	Amphotericin B QC spike levels in plasma		Amphotericin B QC concentration levels μg/ml		Accuracy[c]	%CV[d]
	Free (F-AMP QC)[a]	Liposomal (L-AMP QC)[a]	Free (F-AMP QC)[b]	Liposomal (L-AMP QC)[b]		
1	LLOQ	LLOQ	0.253	0.996	101.80	5.66
2	LLOQ	MQC	0.253	29.990	103.75	2.83
3	LLOQ	HQC	0.253	80.088	106.81	2.50
4	MQC	LLOQ	5.050	0.996	99.39	0.68
5	MQC	MQC	5.050	29.990	91.77	4.39
6	MQC	HQC	5.050	80.088	96.71	1.46
7	HQC	LLOQ	10.100	0.996	101.18	0.77
8	HQC	MQC	10.100	29.990	90.11	6.74
9	HQC	HQC	10.100	80.088	85.05	0.66

[a] Spiked in triplicate.
[b] Nominal concentration.
[c] % deviation of back calculated concentration from nominal concentration, should be from 85% to 115%.
[d] Coefficient of variance.

Table 3b
Separation efficiency—accuracy of L-AMP after separation.

S. No.	Amphotericin B QC spike levels in plasma		Amphotericin B QC concentration levels μg/ml		Accuracy [c]	%CV[d]
	Free (F-AMP QC)[a]	Liposomal (L-AMP QC)[a]	Free (F-AMP QC)[b]	Liposomal (L-AMP QC)[b]		
1	LLOQ	LLOQ	0.253	0.996	103.85	3.31
2	LLOQ	MQC	0.253	29.990	105.58	3.95
3	LLOQ	HQC	0.253	80.088	106.59	3.71
4	MQC	LLOQ	5.050	0.996	111.91	7.88
5	MQC	MQC	5.050	29.990	103.87	8.68
6	MQC	HQC	5.050	80.088	106.87	2.10
7	HQC	LLOQ	10.100	0.996	153.51[†]	11.23
8	HQC	MQC	10.100	29.990	106.03	12.24
9	HQC	HQC	10.100	80.088	112.92	2.25

[a] Spiked in triplicate.
[b] Nominal concentration.
[c] % deviation of back calculated concentration from nominal concentration, should be from 85% to 115%.
[d] Coefficient of variance.
[†] Result was not considered for conclusion derived for separation efficiency.

Table 4a
Stability results of free Amphotericin B in the presence of Acetaminophen and Diphenhydramine hydrochloride.

Stability condition	Storage condition	Level	Free Amphotericin B				
			A[a]	%CV[b]	B[c]	%CV[b]	% Stability[d]
Bench top	Room temperature–4 h	F-AMP LQC	0.78	4.05	0.75	3.52	96.24
		F-AMP HQC	10.27	3.55	9.52	3.18	92.63
Post preparative	After 48 h at 2–8 °C	F-AMP LQC	0.78	2.14	0.77	3.06	98.28
		F-AMP HQC	10.22	1.99	10.69	1.57	104.54
Long-term stability	30 days at −70 °C	F-AMP LQC	0.77	1.32	0.84	1.69	108.70
		F-AMP HQC	10.76	1.30	10.10	2.90	93.88

[a] Mean comparison concentration in μg/ml, $n = 7$.
[b] Coefficient of variance.
[c] Mean stability concentration in μg/ml, $n = 7$.
[d] % mean change, acceptance criteria 100 ± 15%.

Table 4b
Stability results of liposomal Amphotericin B in the presence of Acetaminophen and Diphenhydramine hydrochloride.

Stability condition	Storage condition	Level	Liposomal Amphotericin B				
			A[a]	%CV[b]	B[c]	%CV[b]	% Stability[d]
Bench top	Room temperature–6 h	L-AMP LQC	2.59	7.72	2.83	2.98	109.16
		L-AMP HQC	79.9	3.41	78.00	9.82	97.64
Post preparative	After 48 h at 2–8 °C	L-AMP LQC	2.62	4.54	2.95	12.82	112.72
		L-AMP HQC	68.93	3.23	77.35	6.79	112.22
Long-term stability	30 days at −70 °C	L-AMP LQC	2.51	9.90	2.34	4.88	93.07
		L-AMP HQC	68.87	4.49	74.81	3.22	108.64

[a] Mean comparison concentration in μg/ml, $n = 7$.
[b] Coefficient of variance.
[c] Mean stability concentration in μg/ml, $n = 7$.
[d] % mean change, acceptance criteria 100 ± 15%.

were specificity, matrix effect, recovery, precision and accuracy, stability in plasma and post preparative stability after extraction. Recoveries of F-AMP QCs and L-AMP QCs in the presence of concomitant drugs were unaltered. The results of matrix effect, precision and accuracy, stability tests were within acceptance criteria set for actual validation study. There was no interference observed at the retention times of Amphotericin B and internal standard due to concomitant medicines. The results of stability in plasma in presence of Acetaminophen and Diphenhydramine hydrochloride are tabulated in Tables 4a and 4b. All samples were analyzed as per the procedure and sufficient number of QCs was processed along with subject samples to decide batch acceptance or rejection. Total 100% F-AMP QCs and 98.7% L-AMP QCs were within acceptance criteria. This provides sufficient rationale for acceptance of results of unknown samples. Fig. 6 represents the MRM chromatograms of real subjects for free and liposomal Amphotericin B. $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} (AUC: area under curve, C_{max}: peak plasma concentration) were calculated. The pharmacokinetic parameters were calculated by non-compartment methods using WinNonlin® software version 5.2 (Pharsight

Table 5
Pharmacokinetics parameters of Amphotericin B after infusion of liposomal Amphotericin B (2 mg/kg).

Parameter (unit)	Normalized result for[a]	
	Free Amphotericin B	Liposomal Amphotericin B
C_{max} (μg/ml)	2.66 ± 0.75	29.56 ± 12.03
AUC_{0-t} (μg h ml^{-1})	59.56 ± 23.24	251.10 ± 123.12
$AUC_{0-\infty}$ (μg h ml^{-1})	74.98 ± 20.38	268.78 ± 123.76

[a] Values are mean ± standard deviation for normalized data to 60 kg body weight*.
*Normalization formula: Normalized concentration (μg/ml) = (Analytical concentration (μg/ml) × 60)/weight of subject (kg).

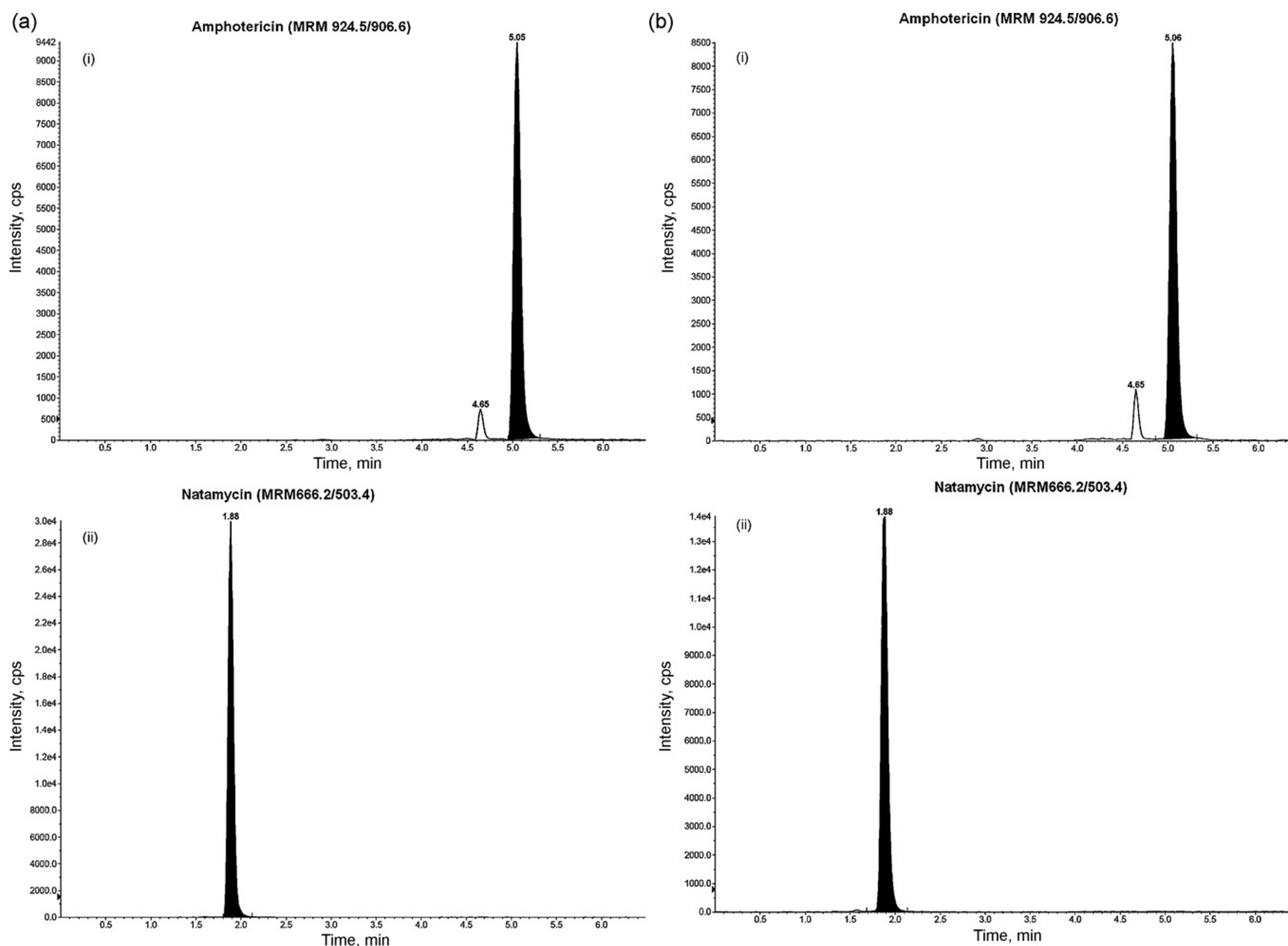

Fig. 6. Real subject chromatograms of (a) free Amphotericin B (1.93 μg/ml) and (b) liposomal Amphotericin B (19.20 μg/ml) at 2.0 h.

corporation USA). The results of the study are presented in Table 5.

4. Conclusion

The LC–MS–MS bioanalytical method developed for quantification of free and liposomal Amphotericin B from plasma is specific, sensitive, robust, precise and accurate. The method is selective enough to separate the two states of Amphotericin B, free and liposomal, in human plasma. It enabled the profiling of free and liposomal Amphotericin B in plasma. The validation data demonstrate good precision and accuracy of the method. The method was robust, without lot-to-lot variation in matrix effect. The recoveries of the extracted samples were higher and consistent over the concentration range selected for determination of free and liposomal Amphotericin B compared to previous reported method [21]. The method was applied successfully to samples from healthy human volunteers dosed with a marketed liposomal Amphotericin B formulation AmBisome. Specificity, selectivity and stability of the method were proven in the presence of concomitant medications, Acetaminophen ER (650 mg) and Diphenhydramine hydrochloride (50 mg) tablets given to subjects. The C_{max} values obtained with the analysis of subject samples justifies the linearity and range selected for assaying free and liposomal Amphotericin B.

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