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Rapid Quantitative Evaluation of Amphotericin B in Human Plasma, by Validated HPLC Method

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Abstract

A simple, rapid, and sensitive HPLC method was developed and validated to quantify Amphotericin B (AmB) in human plasma. AmB was extracted from spiked plasma by simple protein precipitation with methanol. The separation was performed on an XBridge™ C18 (150 × 4.6 mm, 3.5 μm) column, with a mobile phase of acetic acid (0.73%) – acetonitrile (60:40, v/v) and at a flow rate of 1 mL/min. The eluted peak of AmB was monitored at 408 nm with photo-diode array detector (PDA) detector. The calibration curve was found linear in the AmB concentration range of 1000 – 50 ng/mL (r^2>0.99). The inter- and intra-day precisions (%CV) were less than 11.2%. The extraction recoveries were 85-91%. The method developed and validated is simple, rapid (<3 min per injection), sensitive, and reproducible. It potentially can be used for the pharmacokinetic, bioequivalence, and toxicokinetic studies of AmB.

Keywords: Amphotericin B; HPLC; Method development; Validation; Extraction; Human plasma

Introduction

Amphotericin B (AmB), an amphipathic fermentation compound produced by the South American soil gram positive bacterium *Streptomyces nodosus* and is considered as a gold standard for treating severe systemic fungal infections. Long-term usage of AmB leads to high incidence of adverse effects, predominantly nephrotoxicity and infusion-related toxicity. However, several formulations of AmB were developed to reduce the toxicity and to improve the therapeutic efficacy, they still cause considerable amount of toxicity [1,2]. Hence, a careful monitoring of patients under treatment with AmB is required and demands for a simple, rapid, sensitive, and inexpensive method to determine AmB in biological samples like plasma and there by determine its pharmacokinetic, bioequivalence, and toxicokinetic profile.

Several analytical methods have been reported for the determination of AmB in biological samples like plasma using high pressure liquid chromatography (HPLC) [3-16]. However, some of the reported HPLC methods have used salts in their mobile phase [7,12,13], which lowers the life-time of the column by significantly increasing the risk of saturation, breakdown or over pressure in the column. Longer retention times in a few reported methods [4,8,9,15] require more time to analyze the samples and also consume more solvents. Though a reported method indicated short retention time for the elution of AmB, the peak resolution and symmetry is questionable [6].

The aim of this study was to develop and validate a simple, rapid, sensitive and reproducible isocratic HPLC method with a well resolved symmetrical peak of interest for the estimation of AmB in plasma samples. This method can be easily established in research and clinical laboratories for analyzing large number of plasma samples obtained for pharmacokinetic, bioequivalence or toxicokinetic studies after administration of AmB.

Materials and Methods

Reagents and chemicals

AmB USP analytical sample standard was obtained from MP Biomedicals (Santa Ana, CA, USA) and HPLC grade methanol, acetonitrile, dimethyl sulfoxide (DMSO), acetic acid and water were obtained from Fisher (Pittsburg, PA, USA). Drug-free human plasma was purchased from Innovative Research (Novi, MI, USA).

Instrumentation

All chromatographic studies were conducted on validated and qualified equipment. Chromatography was performed with a Waters HPLC Alliance system on an e2695 separations module with a Waters 2998 photo-diode array (PDA) detector (Milford, MA, USA). Samples were injected using a Waters auto injector and the instrument was controlled by use of Empower2® software (Milford, MA, USA). Components were separated on a Waters XBridge™ C18 reversed-phase column (Milford, MA, USA) with 150mm X 4.6 mm dimensions and 3.5 μm particle size. The column was kept thermostatic at 30°C in a Waters column oven (Milford, MA, USA).

Chromatographic conditions

The developed method used for detection and quantification of AmB was an isocratic method using an XBridge™ C18 reversed-phase column with a mobile phase of acetic acid (0.73%)-acetonitrile (60:40, v/v). The flow rate of the solvent was 1.0 mL/min with a runtime of 6 minutes. Samples were injected into the column at a constant volume of 20 μL and a PDA detector at 408 nm was employed to obtain the responses of varied concentrations of AmB. The column was maintained at 30°C.

Statistical methods

Standard calibration curves were plotted using peak areas and

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concentrations of the standard solutions. The data were fit to a linear model using a least squares regression analysis. The back calculated concentrations of the standards and the plasma samples (calculated using regression line) were used to determine the means, standard deviations (SD), and % coefficients of variation (%CV) at each concentration.

Preparation of standards for calibration curve

AmB stock solutions were prepared each day of analysis, by dissolving an accurate amount of USP standard AmB in the mixture of HPLC grade DMSO-methanol (50:50, v/v). The standard concentrations of 1000, 800, 600, 400, 200, 100, and 50 ng/mL were prepared by serial dilution in HPLC grade methanol. Each standard solution was injected (20 µL/injection) in sextuplicate and chromatographed under the described conditions. The method developed for the analysis of AmB was validated by developing a standard calibration curve with the standard concentrations on three different days. A different stock solution prepared each day, respectively. The following parameters were calculated for the validation of the AmB assay; linearity, accuracy, intra-day and inter-day precision, limit of detection (LOD), and limit of quantification (LOQ).

Method for determining linearity and accuracy

The standard dilutions of AmB (1000-50 ng/mL) were injected in series and the peak area responses were recorded. The standard concentrations were plotted against each peak area. A line of least squares regression was constructed, the slope and intercept were calculated and the coefficient of determination was determined for each calibration curve. Accuracy was determined by quantifying standard solutions in sextuplicate followed by the determination of the mean and % nominal.

Ruggedness

Ruggedness of the method developed is evaluated by testing the reproducibility of the AmB peak resolution and its retention time obtained under a variety of normal test conditions, such as different laboratory, different analyst, different instruments, different lots of reagents, and different days.

Method for determining precision, LOD, and LOQ

Precision was calculated in accordance with International Conference on Harmonisation (ICH) guidelines for both repeatability and intermediate precision [17]. R -Galera et al. [6]. Human plasma samples (100 epeatability of the method was determined as intra-day variation and intermediate precision of the assay was determined by inter-day variation [18,19]. In order to determine both the intra-day and inter-day precision, AmB solutions at six different concentrations (1000, 800, 400, 200, 100, and 50 ng/mL) were determined in sextuplicate and mean and % CV were calculated. LOD and LOQ were determined by applying the following formula [20];

$$LOD = 3.3\sigma/m$$

$$LOQ = 10\sigma/m$$

Where, σ is the standard deviation of the intercept of regression line, and m is the slope of the calibration curve.

Plasma extraction and preparation of the samples

The method of extraction was adapted from previously reported protocol by Lopez-Galera et al. [6]. Human plasma samples (100 µL) were spiked with AmB solution (20 µL) to obtain the plasma concentrations of 5, 2, 1, and 0.5 µg/mL and incubated at ambient temperature for 10 min. Spiked drug was extracted by adding 680 µL of methanol to spiked plasma samples, vortex for 30 secs and sonicated for 3 minutes. The samples were then centrifuged at 20000 g for 5 min at 4°C and supernatant was assayed for recovery of AmB in triplicates by using validated HPLC assay. A control sample of plasma was prepared in the similar way but without adding AmB.

Results and Discussion

HPLC method development

An HPLC method was developed to measure AmB concentrations from 1000 to 50 ng/mL in plasma samples by using XBridge™ C18 column and simple mobile phase. Different flow-rates and ratios of the mobile phase were studied in order to obtain a well resolved symmetrical AmB peak with a shorter retention time. The optimal separation was obtained with a mobile phase of acetic acid (0.73%)-acetonitrile (60:40, v/v) and with a flow-rate of 1 mL/min. The AmB peak in the standard solutions indicated a baseline separation with a retention time of approximately 2.7 minutes. The suitability of the method for analysis of AmB was confirmed by analysis of AmB concentration in each spiked plasma sample. Example chromatograms obtained for drug-free human plasma and AmB spiked human plasma are shown in figure 1.
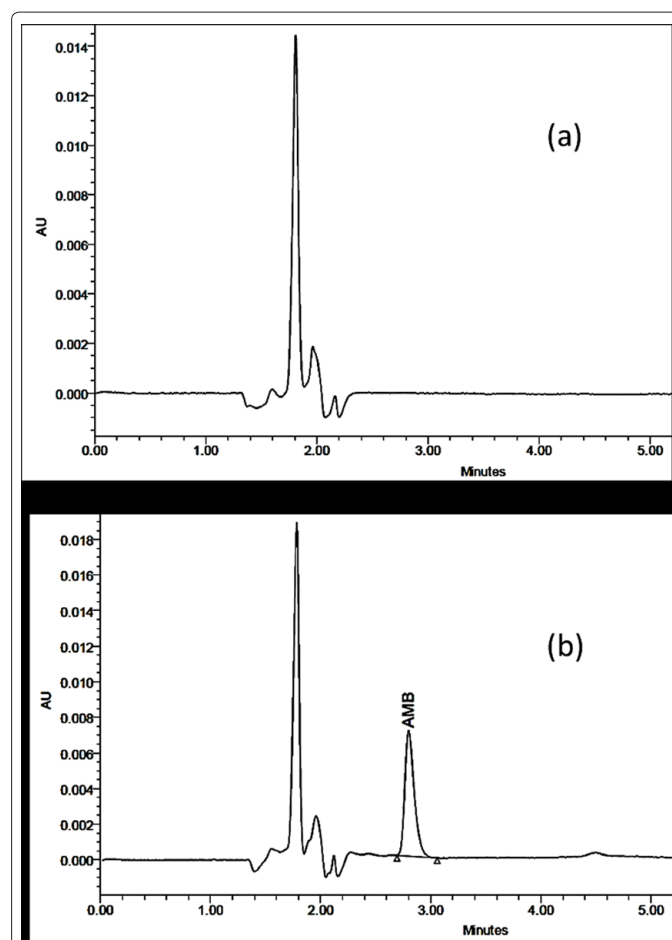

Figure 1: A representative chromatograms of drug-free plasma (a) and AmB spiked human plasma (b), retention time (t_R) =2.7 minutes.

No other peak is eluted with retention time similar to AmB in blank human plasma.

Linearity & accuracy

The linearity of the peak area versus the concentration (amount) was studied in the range of 1000-50 ng/mL for AmB and the results were subjected to statistical analysis using a linear-regression least-squares method. The linear regression data for the calibration curve, as shown in the table 1, is indicative of a significant linear relationship between amount and peak area over the range studied. The calibration curves were found to be linear with a correlation coefficient (r^2) of 0.9964-0.9999. There were no significant inter-day differences between the slopes of the calibration curves. The low values of the standard deviation and standard error of slope indicates lack of deviation from the linearity or analytical bias in the calibration curve.

The accuracy of the method was determined by calculating the mean concentrations and % nominal mean for concentrations 1000 to 50 ng/mL for the three standard curves of AmB (Table 2). The % nominal mean (93.9-111.61%) indicate the method is acceptably accurate.

Precision, LOD & LOQ

Precision was determined, as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of the method was determined as intra-day variation, and intermediate precision was calculated by determining inter-day variation for sextuplicate determinations of AmB at six different concentrations

Linearity range (ng/mL)	50-1000
Regression equation	y=129.33x - 1050.83
Correlation coefficient (r^2) ± SD	0.9981 ± 0.0018
Slope ± SD	129.33 ± 4.04
Standard error of slope	2.33

Table 1: Linear regression data for calibration curve (n=3).

Days	Actual Conc. (ng/mL)	Mean Conc. (ng/mL)	Standard Deviation	Nominal (%)	CV (%)
1	50	52.385	1.48	104.77	2.82
2	50	46.949	1.16	93.9	2.48
3	50	60.806	0.85	111.61	1.39
1	100	101.626	0.98	101.63	0.96
2	100	90.363	1.5	99.36	1.66
3	100	109.939	1.32	109.94	1.2
1	200	196.742	0.85	98.37	0.43
2	200	198.633	1.3	99.32	0.66
3	200	192.351	1.97	96.18	1.02
1	400	399.164	1.47	99.79	0.37
2	400	408.131	1.83	102.03	0.45
3	400	385.919	2.42	96.48	0.63
1	800	796.896	2.07	99.61	0.26
2	800	838.937	2.82	104.87	0.34
3	800	776.791	3.69	97.1	0.47
1	1000	1003.187	5.57	100.32	0.56
2	1000	966.989	3.94	96.7	0.41
3	1000	1024.862	2.64	102.42	0.26

Table 2: Accuracy and intra-day precision of the method for the determination of AmB (n=6).

Actual conc. (ng/mL)	Mean Conc. (ng/mL) (n=18)	CV (%)
50	53.380	11.19
100	100.642	8.29
200	195.908	1.55
400	397.738	2.40
800	804.208	3.33
1000	998.124	2.47

Table 3: Inter-day variability of the method for the determination of AmB.

Plasma Conc. (μg/mL)	Spiked (ng)	Found (ng)	%Recovery (mean ± SD)	CV (%)
5	600	535.38	89.23 ± 1.88	0.27
2	240	204.528	85.22 ± 2.37	0.90
1	120	105.132	87.61 ± 0.52	0.39
0.5	60	54.768	91.28 ± 1.47	1.90

Table 4: Accuracy and recovery of AmB in spiked human plasma (n=3).

(1000 and 50 ng/mL) on three different days for three calibration curves. Calculated values of mean and %CV for determination of repeatability and intermediate precision are listed in tables 2 and 3. The low values of %CV indicate the acceptable repeatability and intermediate precision of the method. The LOD and LOQ of AmB of the developed method were found to be 9.98 and 29.97 ng/mL, respectively. This sensitivity is achieved with just 20 μL of injection volume and can be improved with higher injection volumes.

Ruggedness

Under a variety of normal test conditions as mentioned in the methods section, the AmB peak was found to be well resolved from all other peaks with the retention time of 2.7 minutes, illustrating the ruggedness of the method.

Recovery

Mean recovery of AmB from human plasma was found to be 88% at concentrations of 500 to 5000 ng/mL. Recoveries and the accuracy of the assay in determining AmB in spiked human plasma samples are summarized in table 4. The deviation from theoretical values is under 15% at all concentrations levels studied.

Conclusion

A validated isocratic HPLC method has been developed for the determination of AmB in human plasma samples. The method is simple, rapid, accurate, and precise. Additionally, relatively shorter retention times (<3 min per injection) of AmB allow the analysis of a large number of samples in a short period of time. Therefore, this rapid and inexpensive method should be suitable for the routine analysis of AmB in clinical settings and to evaluate its pharmacokinetic, bioequivalence, and toxicokinetic profile.

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