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Article

Sensitive, Accurate and Simple Liquid Chromatography–Tandem Mass Spectrometric Method for the Quantitation of Amphotericin B in Human or Minipig Plasma

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Amphotericin B (AMB) is still the standard care for systemic fungal infections. This paper describes a sensitive, accurate and simple liquid chromatography–tandem mass spectrometry method to quantify AMB in human or minipig plasma. Samples were prepared through protein precipitation by adding methanol–acetonitrile (1:3, v/v) to either human or minipig plasma. High-performance liquid chromatography separation was conducted on a 10-cm Gemini C18 column with a 7-min gradient of mobile phase comprised of buffer A (0.1% formic acid aqueous solution) and buffer B (methanol–acetonitrile, 2:3, v/v). AMB was detected through multiple reaction monitoring (MRM) with a mass transition of 924.60 → 743.30 and the internal standard paclitaxel was detected through MRM with a mass transition of 854.30 → 286.10. The method had a linear range between 5 and 2500 ng/mL with lower limit of quantitation of 3 ng/mL. The overall recovery was 113 ± 4.06% in human plasma and 94.8 ± 7.38% in minipig plasma. The method has been validated and applied for AMB pharmacokinetic study in both human and minipig plasma.

Introduction

Amphotericin B (AMB; Figure 1A), a polyene antibiotic, is a natural fermentation product of *streptomyces nodusus*. It is one of the most potent drugs for treatment of a variety of systemic fungal infections (1). Various methods for AMB quantitation have been developed in the past for its pharmacokinetic study or drug monitoring purpose. These methods include early microbiological assays, popular high-performance liquid chromatography (HPLC) methods (2–7) and one newly developed liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) method (8). The HPLC method gained popularity because microbiological assays suffer from lack of sensitivity and selectivity. Solid-phase extraction has commonly been employed for sample preparation in these methods (5–8), with protein precipitation used in some cases (2–3).

Compared to HPLC with ultraviolet (UV) detection, LC–MS-MS usually has better selectivity, sensitivity and higher throughput. Recently, an LC–MS-MS method to quantitate both bound and free AMB was reported by Lee *et al.* (8). However, the method involved a long sample preparation procedure using solid-phase extraction. The recovery rate was low and the linear range was narrow. The new method we developed greatly simplifies the sample preparation process, improves the recovery rate and has a wider linear range while still having a limit of quantitation as low as 3 ng/mL. Combining the high selectivity of multiple reaction monitoring (MRM) of tandem mass spectrometry with liquid chromatography and protein precipitation by methanol–acetonitrile for sample preparation was enough to achieve high selectivity. In addition to the simplification of sample preparation, the overall recovery was also greatly improved by using protein precipitation. Paclitaxel (Figure 1B) was selected as internal standard (IS) in part because of its similar retention time in the LC conditions and similar molecular weight to the analyte.

This method was developed in support of pre-clinical and clinical studies of an antifungal formulation of AMB for the topical, intranasal treatment of chronic sinusitis, in which the AMB levels are typically lower than 10 ng/mL for minipig, and less than 30 ng/mL in human subjects.

Material and Methods

Chemicals

AMB was purchased from United States Pharmacopeia (Rockville, MD). The IS, paclitaxel, was obtained from Sigma Chemical Co. (St. Louis, MO). Human plasma and minipig (Göttingen) plasma were from Biochemed Services (Winchester, VA); HPLC-grade methanol and acetonitrile were both from EMD Chemicals (Gibbstown, NJ). ACS-grade formic acid was from Fisher Scientific (Fair Lawn, NJ). Deionized water was prepared using a Purelab water purification system (Siemens Water Technologies, Munich, German).

Liquid chromatographic conditions

The Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) consisted of two LC-10ADvp delivery pumps, a Series 2000 vacuum degasser (Perkim Elmer, Waltham, MA), an SIL-HTC autosampler and an SCL-10Avp system controller. HPLC separation was conducted on a 5-μ Gemini C18 column (4.6 × 100 mm, Phenomenex, Torrance, CA) column using a 7-min gradient of mobile phase comprised of buffer A (0.1% formic acid aqueous solution) and buffer B (methanol–acetonitrile, 2/3, v/v). The 7-min gradient at a total flow rate of 0.5 mL/min consisted of 1.5 min of 80% buffer B, 0.5 min for buffer B to decrease to 20%, 3 min of 20% buffer B, then 0.5 min to increase to 80% buffer B and finally 1.5 min of 80% buffer B. The column was kept at ambient temperature and the autosampler temperature was kept at 8°C. The sample injection was set at 1 μL.

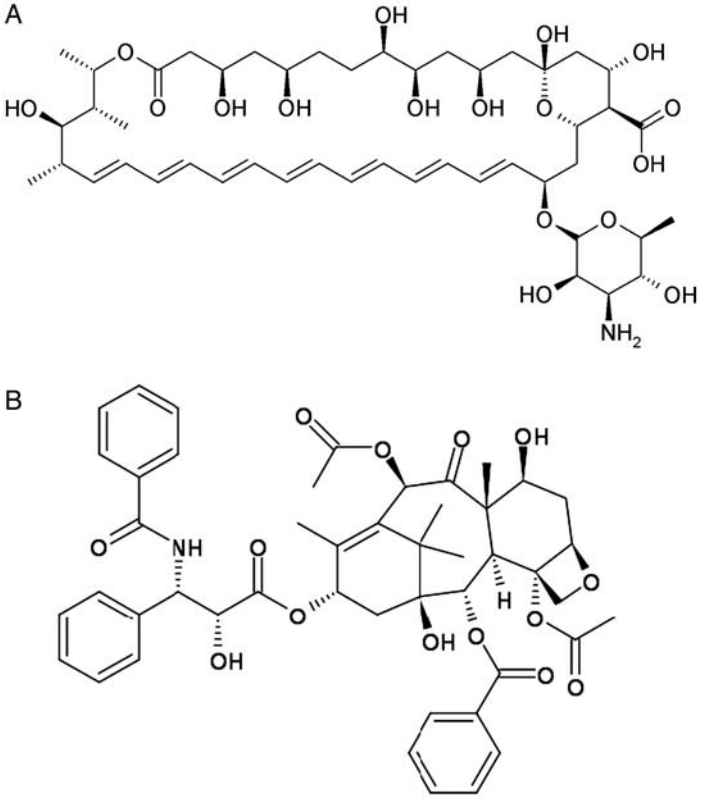

Figure 1. Chemical structures of AMB (A) and paclitaxel (IS) (B).

Mass spectrometric conditions

A Sciex API 4000 triple-quadrupole mass spectrometer (AB Sciex, Foster City, CA) with a TurboSpray ion source was used to analyze samples. Nitrogen gas was used as nebulizer, collision and curtain gas. AMB and IS were detected by tandem mass spectrometry using MRM with a mass transition of m/z 924.60 → 743.30 and 854.30 → 286.10, respectively. After optimization, the working MS parameters were: curtain gas, 20; collision gas, Gas 1 (nebulizer gas) flow rate 55; Gas 2 (heater gas) flow rate 35; ESI voltage, 4,500 V; collision energy (CE), 30.6; collision cell exit potential (CXP), 18.16 V; ionization mode, positive; TurboSpray source temperature, 450°C; declustering potential (DP), 56.2 V; entrance potential (EP), 11 V. All the data were acquired using Analyst 1.4 software (AB Sciex).

Preparation of samples by protein precipitation

Plasma samples (150 μL each) including standards and quality control (QC) samples were added to a 475-μL IS working solution (0.2 μg/mL paclitaxel in methanol–acetonitrile). The samples were votexed for 30 s on a Genie 2 vortexer (Fisher Scientific) and placed in a −20°C freezer for 45 min followed by 10 min centrifugation at 1,000 g on an Eppendorf 5417C Microcentrifuge (Eppendorf, Westbury, NY). The supernatants were then transferred to a 96-well plate for analysis.

Preparations of standard, QC samples and IS

Stock AMB solution (0.1 mg/mL) was prepared by dissolving 5 mg of AMB in 50 mL of methanol with a few drops of dimethyl sulfoxide (DMSO). Stock AMB solution was diluted with methanol to achieve an intermediate stock concentration of 10 μg/mL for standard solution preparation. Eight standards, plus one limit of quantification (LOQ) and one limit of detection (LOD) sample containing AMB concentrations of 2,500, 1,000, 500, 250,100, 50, 25, 5 and 3 (LOQ) and 1 ng/mL (LOD), respectively, were prepared by proper dilution with human or minipig plasma. QC sample solutions of QC-high (500 ng/mL), QC-med (250 ng/mL) and QC-low (50 ng/mL) were prepared in a similar way as the standards for precision study. The other set of QC samples at QC-high (1,000 ng/mL), QC-med (500 ng/mL) and QC-low (100 ng/mL) were prepared for stability study, also in a similar way. The set of eight standards in methanol solution for recovery study was prepared by dilution of AMB intermediate stock solution at a concentration of 10 μg/mL with methanol. IS solution was prepared by dissolving appropriate amounts of paclitaxel in methanol–acetonitrile (3:1, v/v) and further diluted with methanol–acetonitrile (1/3, v/v) to achieve a final concentration of 0.2 μg/mL.

Method validation

Accuracy

The accuracy of the method was demonstrated by the recovery of AMB in a concentration range of 5 to 2,500 ng/mL. The

recovery was performed in duplicate at the approximate concentrations of 5, 25, 50, 100, 250, 500, 1,000 and 2,500 ng/mL in both human and minipig plasma.

Linearity and range

Solutions prepared as described previously at the approximate concentrations of 5, 25, 50, 100, 250, 500, 1,000 and 2,500 ng/mL were examined (calculation from the same raw data set). Within the studied range, separate peak areas were plotted versus the theoretical concentration. Correlation coefficients from each curve were used to evaluate the linearity.

Precision

Six sample solutions at 50 to 500 ng/mL concentration levels (duplicate at each level) were prepared (as described previously) and tested according to the procedure for QC sample testing. Percent relative standard deviation (%RSD) of the sample recoveries was calculated to evaluate the method precision.

Specificity

The specificity was defined as no inference at AMB retention time from human plasma or minipig plasma and no inference from IS under current sample preparation procedures and LC–MS-MS conditions. This was to demonstrate AMB and IS signal levels at their retention times in the following six samples; human or minipig plasma double blank, human or minipig plasma blank and standard human or minipig plasma solution in comparison with AMB at a concentration level of 10 ng/mL.

Recovery rate by protein precipitation

AMB recovery rate in human or minipig plasma by protein precipitation in sample preparation was demonstrated by the recovery of AMB in a concentration range from 5 to 2,500 ng/mL. The recovery was performed at the approximate concentrations of 5, 25, 50, 100, 250, 500, 1,000 and 2,500 ng/mL in methanol, human plasma and minipig plasma. Recovery rate in human or minipig plasma was evaluated by comparing them with those in methanol.

Determination of LOD

LOD standard solution in both human and minipig plasma was run in triplicate. After assuring that the height of each analyte peak was greater than 3, the signal-to-noise (S/N) ratio (by dividing the signal of the analyte peak by the noise) was calculated.

Determination of LOQ

LOQ standard solution in both human and minipig plasma was run six times. After assuring that the height of each analyte peak was greater than 6, the S/N ratio (by dividing the signal of the analyte peak by the noise) was calculated. The %RSD of peak area for the six determinations was determined.

Solution stability

Two types of solution stability, solution storage stability and solution freeze/thaw stability, were examined.

Solution storage stability

Sample solutions of QC-high (1,000 ng/mL),d QC-med (500 ng/mL) and QC-low (100 ng/mL) were stored at −20°C or −80°C. Each solution was analyzed in triplicate initially for AMB and after three, 15 or 30 days against freshly prepared standards. Solution stability was run using the same instrument, column and mobile phase preparations where feasible.

Solution freeze/thaw stability

Duplicate sample solutions of QC-high (1,000 ng/mL), QC-med (500 ng/mL) and QC-low (100 ng/mL) were tested for three freeze/thaw cycles between frozen temperature (−20°C or −80°C) and room temperature. Solution stability was run using the same instrument, column and mobile phase preparations where feasible.

Results and Discussion

Accuracy

The method accuracy data in both human plasma and minipig plasma are summarized in Table I. Based on the duplicate experiments, the mean accuracy ($n = 16$) for human plasma and minipig plasma was 100.0 with an RSD of 8.51% and 100.0 with an RSD of 12.6%, respectively. The assay had a very satisfactory accuracy over the range of 5–2,500 ng/mL; additionally, the accuracy decreased a little at low concentrations near LOQ (3 ng/mL).

Linearity and range

The ratio of analyte peak area/IS peak area (y) were plotted against the theoretical analyte concentration (x) to generate calibration curves. Correlation coefficients (r^2) from analytes over two calibration curves in each matrix were all greater than 0.998. A representative calibration curve is shown in Figure 2. The range of calibration curves, 5 to 2,500 ng/mL, was chosen to facilitate the quantitation of the analyte in clinical samples.

Table I
Method Accuracy in Both Human and Minipig Plasma Standard Samples

Sample		Human plasma		Minipig plasma	
Standard	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)
1_1	5	4.6	92	6.34	126.8
1_2	5	6.29	125.8	6.08	121.6
2_1	25	23.5	94	24.3	97.2
2_2	25	27.4	109.6	27.3	109.2
3_1	50	50.1	100.2	42.2	84.4
3_2	50	48.9	97.8	44.7	89.4
4_1	100	99.1	99.1	79.6	79.6
4_2	100	87	87	86.1	86.1
5_1	250	236	94.4	237	94.8
5_2	250	243	97.2	245	98
6_1	500	492	98.4	532	106.4
6_2	500	502	100.4	489	97.8
7_1	1,000	1,030	103	1,070	107
7_2	1,000	1,010	101	1,030	103
8_1	2,500	2,530	101.2	2,510	100.4
8_2	2,500	2,480	99.2	2,440	97.6
		Mean	100.0	Mean	100.0
		%RSD	8.51	%RSD	12.6

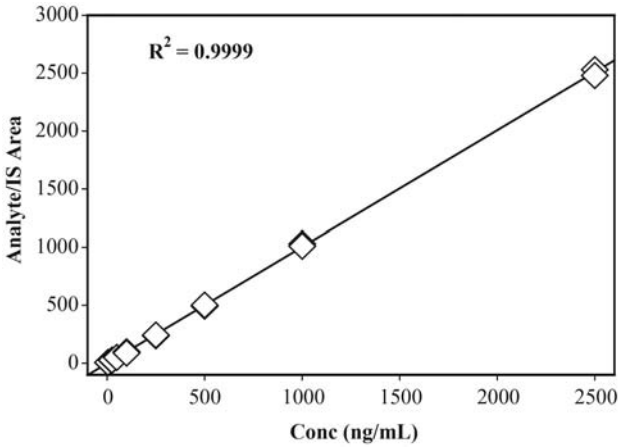

Figure 2. A representative AMB calibration curve with human plasma.

Table II
Method Precision in Human and Minipig Plasma in QC Samples

Sample matrix		Human plasma		Minipig plasma	
QC samples	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)
QCL_1	50	52.3	104.6	45.6	91.2
QCL_2	50	51.9	103.8	48.4	96.8
QCM_1	250	260	104	242	96.8
QCM_2	250	243	97.2	251	100.4
QCH_1	500	474	94.8	485	97
QCH_2	500	519	103.8	491	98.2
Mean			101.4		96.7
%RSD			4.18		3.15

Precision

The precision data of both matrixes are summarized in Table II. Based on the six QC samples used, the precision was 4.18 and 3.15% in human and minipig plasma, respectively. These data demonstrated that this method was highly precise in both matrixes.

Specificity

MRM with mass transitions of m/z 924.60 → 743.30 and 854.30 → 286.10 was used to detect AMB and IS, respectively. There was no significant interference from either matrix at AMB retention time approximately 3.6 min (Figures 3 and 4). IS was eluted at approximately ~3.6 min and did not interfere with the analyte detection either.

Sample recovery rate by protein precipitation

The sample recovery rate by protein precipitation in two matrixes was compared to that in methanol (Table III). The recovery rate was 113 ± 4.06% in human plasma and 94.8 ± 7.38% in minipig plasma (both against recovery rate in methanol) over the range for the eight standard concentrations. This rate is generally much better than that of solid-phase extraction. Notably, the recovery rate in human plasma was higher than in minipig plasma. This could be a result of the difference between the two matrixes that may be largely attributed to a drug-protein binding effect.

LOD and LOQ

LOD samples from both matrixes were run in triplicate. All the six samples had S/N ratios well above 3. The LOD in both

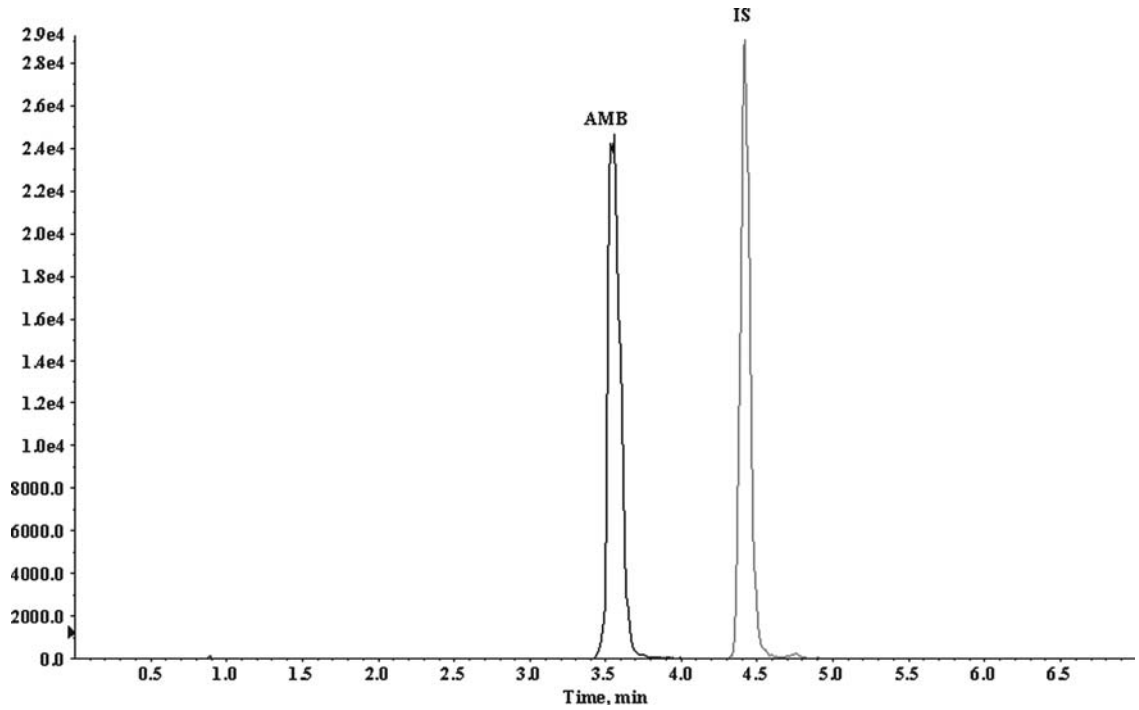

Figure 3. MS chromatogram of AMB (500 ng/mL) spiked in human plasma.

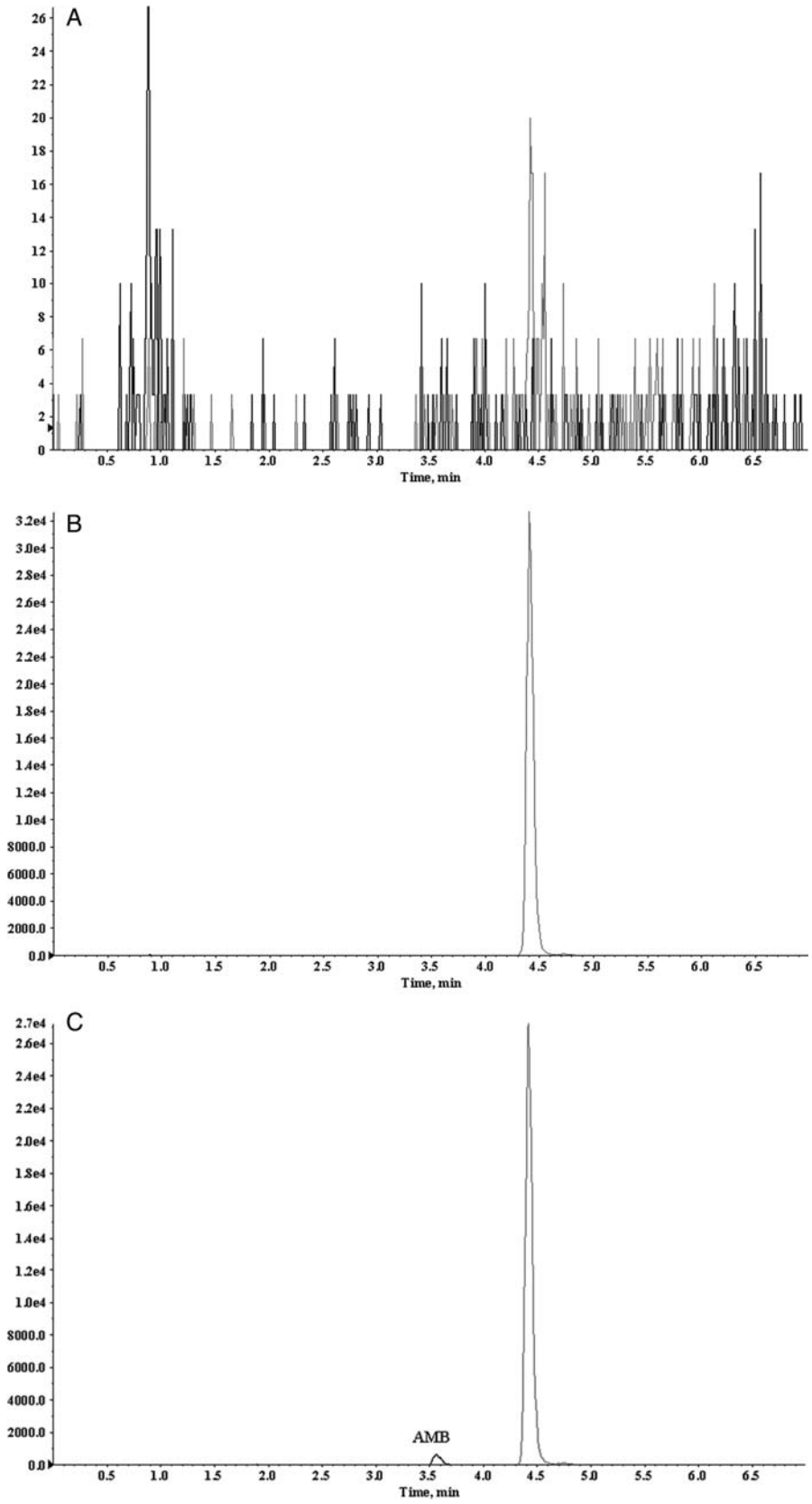

Figure 4. Representative MRM chromatograms in human plasma (A–C) and minipig plasma (D–F): human plasma double blank (A); human plasma blank (B); human plasma with 100 ng/mL AMB (C); minipig plasma double blank (D); minipig plasma blank (E); minipig plasma with 50 ng/mL AMB (E). IS is in red or retention time ~4.5 min; AMB is in blue or retention time ~3.6 min.

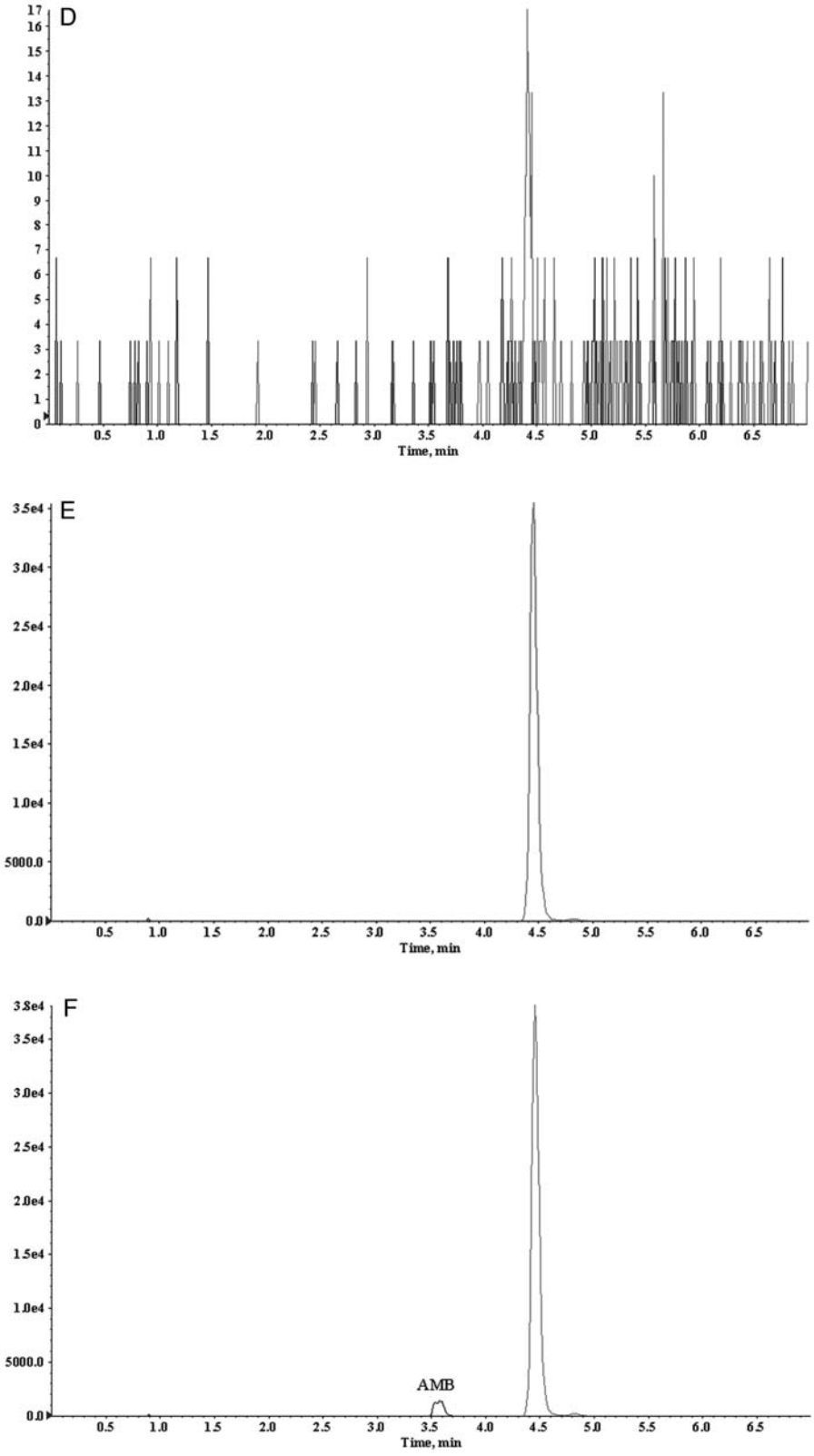

Figure 4. (Continued)

Table III
Recovery Rate in Human Plasma and Minipig Plasma

Sample matrix		In methanol		Human plasma		Minipig plasma	
Standard	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)
1	5	4.87	97.4	5.55	111	4.2	84
2	25	25.6	102.4	27.8	111.2	21.9	97.6
3	50	48.4	96.8	55.3	110.6	43.2	86.4
4	100	92	92.0	109	109.0	80.2	80.2
5	250	231	92.4	253	101.2	235	94.0
6	500	450	90.0	519	103.8	482	96.4
7	1,000	948	94.8	1,100	110.0	999	99.9
8	2,500	2,500	100.0	2,620	104.8	2,420	96.8
Mean			95.7		107.7		90.7
%RSD			4.42		3.59		7.78
Percent versus AMB in methanol			100		113.0		94.8
Adjusted %RSD*					4.06		7.38

*Based on the recovery of AMB in methanol.

Table IV
Determination of LOQ in Human and Minipig Plasma

Matrix	Human plasma		Minipig plasma	
	Calculated concentration (ng/mL)	Accuracy (%)	Calculated concentration (ng/mL)	Accuracy (%)
LOQ3-1	2.4	79.0	3.5	117.0
LOQ3-2	3.2	107.0	3.0	101.0
LOQ3-3	2.4	79.7	3.8	127.3
LOQ3-4	3.5	116.0	3.3	111.3
LOQ3-5	3.5	117.7	2.8	96.0
LOQ3-6	2.8	91.7	3.3	110.3
	Mean	98.5	Mean	110.5
	%RSD	16.2	%RSD	9.2

matrixes was 1 ng/mL. LOQ data are summarized in Table IV. The RSDs were 16.2% for human plasma and 9.2% for minipig plasma. The method performed better in minipig plasma samples than in human plasma samples at the LOQ level.

Solution stability

Solution stability data under three freeze/thaw cycles and short-term storage in two different temperature conditions are summarized in Table V. The mean accuracy ($n = 3$) of different conditions was compared to that of freshly prepared standards to generate mean accuracy difference (% diff. in Table V). These data show that all human plasma samples under any of the conditions examined were stable and with $\pm 6.0\%$ mean accuracy difference against freshly prepared samples. In minipig plasma, the number was $\pm 10\%$. The conditions (3, 15 and 30 days of storage in a −20 or −79°C freezer and three freeze/thaw cycles from −20 or −79°C) covered most of the sample storage and sample preparation during the assay. Data also show that samples were more stable under storage at −79°C than −20°C in both matrixes.

Application

This LC–MS-MS method has been successfully used to determine the pharmacokinetic profile of ABM in both human and minipig plasma.

Table V
AMB Sample Solution Stability

Temperature			−20°C		−79°C	
Matrix	Conditions	Nominal concentration (ng/mL)	Mean value (%) ($n = 3$)	Change from Day 0 (%)	Mean value (%) ($n = 3$)	Change from Day 0 (%)
Human plasma	Day 0	100	114.0			
		500	102.5			
		1,000	106.7			
	Freeze/thaw	100	119.3	5.3	112.6	−1.4
		500	102.3	−0.2	103.5	1.0
		1,000	110.0	3.3	103.3	−3.4
	Day 3	100	117.7	3.7	114.2	0.2
		500	103.6	1.1	103.3	0.8
		1,000	101.7	−5.0	105.3	−1.4
	Day 15	100	108.7	−5.3	111.7	−2.3
		500	102.9	0.4	105.4	2.9
		1,000	108.0	1.3	106	−0.7
	Day 30	100	111.3	−2.7	118.7	4.7
		500	99.3	−3.2	103.6	1.1
		1,000	100.8	−5.9	102.3	−4.4
Minipig plasma	Day 0	100	120.7			
		500	102.6			
		1,000	114.3			
	Freeze/thaw	100	126.3	5.6	117	−3.7
		500	108.7	6.1	106.9	4.3
		1,000	106.3	−8.0	110.3	−4.0
	Day 3	100	115.3	−5.4	120.3	−0.4
		500	103.7	1.1	103.1	0.5
		1,000	104.3	−10.0	110.7	−3.6
	Day 15	100	115.7	−5.0	119.3	−1.4
		500	101.1	−1.5	101.6	−1.0
		1,000	108.3	−6.0	112	−2.3
	Day 30	100	119.0	−1.7	118.3	−2.4
		500	102.5	−0.1	102.7	0.1
		1,000	106.3	−8.0	113.7	−0.6

Although protein precipitation as a sample cleanup procedure is generally simpler, more economic and has a higher recovery rate than solid-phase extraction, it challenges the selectivity of the LC–MS-MS system. The method suffered from lack of sensitivity due to the interference from indigenous components of the matrixes when an isocratic mobile phase was used. A unique mobile phase gradient was then developed to separate the interfering components from the analyte. Approximately 17 times more sensitivity (from 50 to 3 ng/mL) was achieved by applying the LC gradient. Matrix effect was also evident in the method developing process. The method

performed much better in minipig plasma than in human plasma at low concentration near the LOQ. However, human plasma sample seemed to have a higher recovery rate with the sample preparation procedure (protein precipitation) than minipig plasma samples. It is possible that protein precipitation clears up minipig plasma samples better than human plasma samples by precipitating out more endogenous components and the analyte, which could explain both the better performance around LOQ and the lower recovery rate in minipig plasma.

Conclusion

A sensitive, accurate and simple LC–MS-MS method has been developed and validated to assay AMB in human or minipig plasma. The sample preparation is fast, economic and has been greatly simplified with the help of a unique liquid chromatography mobile phase gradient. The method has a lower limit of quantitation of 3 ng/mL and a linear range of 5–2,500 ng/mL. It has a much higher overall recovery rate ($113 \pm 4.1\%$ in human plasma and $94.8 \pm 7.4\%$ in minipig plasma) than methods with solid-phase extraction as the sample preparation procedure. It has been successfully applied to pharmacokinetic and toxicokinetic studies in both human and minipig plasma.

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