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96-Well liquid–liquid extraction liquid chromatography-tandem mass spectrometry method for the quantitative determination of ABT-578 in human blood samples

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Abstract

We report here a quantitative method for the analysis of ABT-578 in human whole blood samples. Sample preparation was achieved by a semi-automated 96-well format liquid–liquid extraction (LLE) method. Aluminum/polypropylene heat seal foil was used to enclose each well of the 96-well plate for the liquid–liquid extraction. A liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) method with pre-column regeneration was developed for the analysis of sample extracts. Selective reaction monitoring (SRM) of the mass transitions *m/z* 983–935 and *m/z* 931–883 was employed for the detection of ABT-578 and internal standard, respectively. The ammonium adduct ions $[M + NH_4]^+$ generated from electrospray ionization were monitored as the precursor ions. The assay was validated for a linear dynamic range of 0.20–200.75 ng/ml. The correlation coefficient (*r*) was between 0.9959 and 0.9971. The intra-assay CV (%) was between 1.9 and 13.5% and the inter-assay CV (%) was between 4.7 and 11.3%. The inter-assay mean accuracy was between 86.4 and 102.5% of the theoretical concentrations.

Keyword: ABT-578

1. Introduction

Percutaneous transluminal coronary angioplasty is designed to restore normal blood flow in vessels narrowed by vascular disease and is currently performed >1,000,000 times annually worldwide. However, due to the inherent response of bare metal stenting, characterized by growth factor-induced neointimal proliferation of vascular smooth muscle cells, re-occlusion (restenosis) of the vessel occurs in more than 30% of those patients.

The macrolide natural product rapamycin (sirolimus) has shown remarkable efficacy in animal models of autoimmune disease and allograft rejection and more recently in human transplantation trials. Implantation of a rapamycin eluting stent can effectively prevent intimal hyperplasia [1–3]. ABT-578, Fig. 1, is a rapamycin analog that is under development by Abbott Laboratories for the treatment of restenosis. Similar to rapamycin, ABT-578 forms a molecular complex in blood cells. A validated bioanalytical assay for the measurement of ABT-578 concentration in whole blood samples was required for the assessment of the pharmacological effect of ABT-578.

Currently, liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) technology is widely used for bioanalytical analysis of drug substances in biological matrices [4,5]. Liquid–liquid extraction (LLE) is one of the preferred sample preparation techniques. Analytes are extracted based on the partitioning between the aqueous/blood phase and the organic extraction solvent phase. LLE generally provides cleaner extracts than the solid phase extraction (SPE) sample preparation process. LLE has typically been performed manually in individual test tubes with large solvent volumes. This constituted a challenge for automating the LLE process to match the throughput of LC-MS/MS analysis. Recently a number of papers have been published describing the success of 96-well format LLE for high throughput analysis of plasma and urine samples [6–18]. The 96-well format LLE was also reported for the analysis

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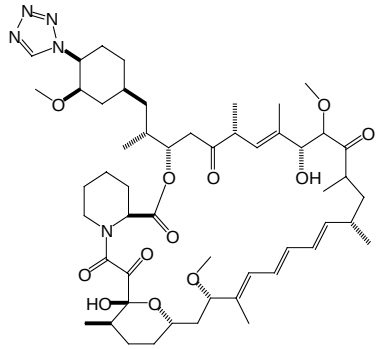

Fig. 1. Structure of ABT-578.

of blood samples [19]. Automated liquid handling was integrated into the sample preparation procedure to improve efficiency of liquid transfer. LLE was completed in individual tubes arranged in a rack [8,13,16] or in a 96-well plate either enclosed with a plate cap mat [6,7,9–11,14] or with aluminum/polypropylene heat seal foil [17,18]. LLE using diatomaceous earth plates in the 96-well format has also been reported [12,15].

In this paper, we are reporting a methodology and results of implementing LLE for the quantitative analysis of ABT-578 in blood samples in a 96-well format. Aluminum/polypropylene heat seal foil was used to enclose each well of the 96-well plate and will be discussed in detail. An isocratic chromatographic method with pre-column regeneration was developed for LC-MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile, ethyl acetate and hexanes, HPLC grade, were purchased from EM Science (Gibbstown, NJ, USA). Formic acid, ACS grade (88%), was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate, ACS grade, was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was provided by a Millipore (Bedford, MA, USA) Milli-Q unit. The reference standard of ABT-578 and the internal standard (IS), rapamycin, were provided by Abbott Laboratories (Abbott Park, IL, USA). Human whole blood with EDTA as the anticoagulant was purchased from Biological Specialties Corporation (Colmar, PA, USA).

2.2. Instrumentation

Blood solutions were transferred using a hand-held pipette from Finnpipette (Needham Heights, MA, USA). A Hamilton automated liquid handler (Reno, NV, USA) was used for reagent transfer. Multi-channel hand-held electronic pipettes from BioHit (Helsinki, Finland), an Advanced Biotechnologies Ltd. (Rochester, NY, USA) heat sealer controlled by a Cole-Parmer (Vernon Hills, IL, USA) heat controller, a Branson (Danbury, CT, USA) sonicator, a VWR (Chicago, IL, USA) vortexer and a Jouan (Winchester, VA, USA) centrifuge were also utilized during sample preparation. The HPLC system included a Shimadzu (Kyoto, Japan) LC-10 AD HPLC Pump, a Shimadzu SIL-10 A XL autosampler, Shimadzu SCL-10 A LC system controller, an HP 1050 backwash LC pump from Agilent Technology (Palo Alto, CA, USA) and a Keystone Scientific (Bellefonte, PA, USA) column heater. The valves used to control LC flow between mass spectrometer inlet and waste line and for the pre-column backwash were from Valco Instruments (Houston, TX, USA). The API 3000 mass spectrometer and computer control system were from PE Sciex (Toronto, Ont., Canada). MassChrom™ version 1.1.1 was used as the data acquisition software. MacQuan™ version 1.6 was used to process the data.

2.3. Chromatographic conditions

The HPLC elution mobile phase was composed of 25 mM ammonium acetate and 0.03% (v/v) formic acid in the solvent mixture of 80/20 (v/v) methanol/water. A flow rate of 0.4 ml/min was used for sample analysis. A 2.0 mm × 100 mm Pharma C18-B, 5 μm, 120 Å column from BHK (Naperville, IL, USA) was used as the analytical column. A 2 mm × 10 mm ODS−P, 5 μm, 120 Å from BHK (Naperville, IL, USA) was used as the pre-column. The analytical column was maintained at a temperature of 50 °C and the injection volume was 40 μl. The elution mobile phase was used as the injector wash solution and the injector was rinsed with 2 ml of wash following each injection. A six-port valve configuration described previously [20] was used to allow for off-line washing of the pre-column with backwash mobile phase (95/5 (v/v) acetonitrile/water), followed by reconditioning with elution mobile phase after each sample injection. The valve was switched to the pre-column back wash position immediately following elution of the analytes to the analytical column. Pre-column back wash was performed simultaneously with elution of the analytes from the analytical column.

2.4. MS/MS detection

LC detection was obtained using a PE Sciex API 3000 triple quadrupole mass spectrometer with a turbo ionspray® ionization source operated in the positive ion mode. The computer control system was Masschrom (Version 1.1.1). The spray voltage was 3000 V, source temperature was 300 °C, nebulizer gas setting was 15, and the curtain gas setting was 10. The other parameters of the state file were optimized by infusing the analyte via a tee connection with the elution mobile phase at a flow rate of 400 μl/min. The selective reaction monitoring (SRM) channel for ABT-578 was *m/z* 983 → 935. The SRM channel for the internal standard was *m/z* 931 → 883.

2.5. Preparation of standard and QC samples

Separate weighings of the reference standard were used to prepare two stock solutions. One stock solution was used to prepare working solutions for the calibration standards. The second stock solution was used to prepare working solutions for the QC samples. Working solutions were prepared by diluting the stock solution of the analyte with 1:1 (v/v) acetonitrile:water. Nine standard levels; 0.20, 0.50, 1.00, 2.01, 8.03, 12.05, 20.08, 60.23, 200.75 ng/ml, were prepared by adding the appropriate volume of working solution into normal human blood with ethylene diamine tetraacetic acid (NHB-EDTA). Four QC levels; 0.55, 2.37, 4.75, 158.26 ng/ml, were prepared by adding the appropriate volume of working solution into NHB-EDTA. Standards and QCs were aliquoted into 5 ml polypropylene vials and stored in an ultra-low temperature freezer set at −70 °C.

2.6. Sample preparation

Standards, QCs and other validation evaluation samples were thawed at room temperature and mixed to ensure homogeneity. One hundred microliters (100 μl) of the internal standard solution were added to each well of the 2.2 ml Marsh Bio Products (Rochester, NY, USA) 96-well plate, except the well designated for the blank. One hundred microliters (100 μl) of freshly prepared 4:1 (v/v) methanol:100 mM ammonium acetate solution were added to each well of the 96-well plate. Three hundred microliters (300 μl) of the calibration standards, QCs, validation evaluation samples, and blanks (drug free blood) were transferred to the appropriate wells of the 96-well plate. Solutions were mixed by aspirating and dispensing 300 μl in each well 5 times. Six hundred microliters (600 μl) of ethyl acetate were added to each well of the 96-well plate. Six hundred microliters (600 μl) of hexanes were added to each well of the 96-well plate. The 96-well plate was sealed with polypropylene/aluminum film from Marsh Bio Products using the heat sealer. The 96-well plate was allowed to sit at room temperature for 45 min, was then sonicated for 5 min, and was then shaken for approximately 5 min using the multi-tube vortexer. The 96-well plate was then centrifuged at 4000 rpm for 10 min at approximately 10 °C. The heat-seal was punctured and 900 μl of the upper (organic) layer of the solution in each well were transferred to the corresponding well of a new 1.2 ml Orochem (Westmont, IL, USA) 96-well plate. The organic extract was evaporated to dryness under a stream of room temperature nitrogen. The dried extracts were reconstituted with 50 μl of the HPLC elution mobile phase and 50 μl of water. The 96-well plate containing the reconstituted extracts was sealed with a cap mat from Orochem Technologies (Westmont, IL, USA) and was shaken for approximately 3 min using the multi-tube vortexer. Forty microliters (40 μl) of the solution in each well were consecutively injected into the LC-MS/MS for analysis.

2.7. Validation

The validation experiments were designed with reference to "Guidance for Industry-Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States [21]. To evaluate the precision and accuracy of the method three runs were performed on three separate days. Each run consisted of a set of calibration standards, six replicates of each QC concentration, six replicates of the LLOQ (lower limit of quantitation) evaluation sample, a blank, and a zero (blank + IS). The stability of the blood samples under various conditions was evaluated as part of the validation. The major items tested during method validation are discussed in the following sections.

3. Results and discussion

3.1. Sample preparation

ABT-578 is hydrophobic which allows for good extraction using a liquid–liquid technique. The extraction was carried out in the 96-well plate by using the Hamilton automated liquid handler and the Advanced Biotechnologies Ltd. heat sealer. This method ensures accurate addition of the internal standard and treatment reagents as well as accurate transfer of the organic layer following centrifugation. Because ABT-578 and the internal standard form molecular complexes in the red blood cells, lyses was necessary. The volume of methanol used for lyses was optimized to break the cell wall without causing significant protein precipitation. Although the mixture of blood and methanol became opaque after the methanol was added, the aqueous portion become clear after centrifuging. The ratio of hexane and ethyl acetate as the extraction solvent was also optimized to achieve maximum extraction efficiency. The method allows a single analyst to prepare multiple batches in a single day increasing the number of samples that can be analyzed in a day and decreasing the time between sample collection and sample processing. The limit to this method is the volume of the wells in the 96-well plate. A 2.2 ml plate was used for this method which limits the pre-extraction volume to approximately 1.8 ml, the extra space being necessary to ensure a proper seal.

The amount of time the heat sealer was allowed to remain in contact with the heat seal film was optimized to give a consistently good seal. If the heat sealer was removed early, the film did not form a good bond with the 96-well plate, which led to cross-contamination between wells. If the heat sealer was allowed to remain in contact with the film for too long, the extraction solvent would begin vaporizing and this would prevent proper bonding between the film and the 96-well plate. Multiple types of aluminum/polypropylene heat seal films were tested. It was found that an aluminum/polypropylene film with a thin layer of aluminum and a thicker layer of polypropylene gave the best results.

The thicker layer of polypropylene on the film meant there was more material available to bond to the surface of the 96-well plate. The thinner layer of aluminum allowed for a better transfer of heat from the heat sealer to the polypropylene layer of the film and the polypropylene making up the 96-well plate. The thin aluminum also allows a better visual inspection of seal integrity.

3.2. LC-MS/MS detection

The electrospray ionization (ESI) mass spectra obtained by infusing ABT-578 and the internal standard in 1:1 (v/v) acetonitrile:water via a tee junction with LC elution mobile phase at the flow rate of 0.4 ml/min are shown in Fig. 2A and B, respectively. The sodium and ammonium adducts of the molecular ion were observed for the analyte (*m/z* 988, *m/z* 983) and the potassium, sodium and ammonium adducts of the molecular ion were observed for the internal standard (*m/z* 952, *m/z* 936, *m/z* 931). There are some ions in the MS spectra that correspond to the product ions in the MS/MS spectra. This is likely due to fragmentation occurring in the source. The ammonium adduct ions were selected as the precursor ions. Under optimized fragmentation conditions several product ions were observed for each compound and the most intense was selected as the product ion to be monitored. The fragment ion at *m/z* 935 is the main product ion of ABT-578, Fig. 2C, and the fragment ion at *m/z* 883 is the main product ion of the internal standard, Fig. 2D.

Chromatographic peak shape and retention times were optimized by heating the column to 50 °C. Analysis using a column at room temperature led to poor peak shape and long retention times. Heating the column greatly improved the chromatographic peak shape and reduced the column pressure. No significant degradation of column performance was observed during the validation of this method.

The LC-MS/MS chromatograms presented in Fig. 3 are from the high standard, 200.75 ng/ml ABT-578 and 1 μg/ml IS, low standard, 0.20 ng/ml ABT-578 and 1 μg/ml IS, and an extracted blank. Total run time for each sample was 8.5 min. Mass spectrometric detection was initiated 2.5 min after injection and was terminated 7 min after injection. The actual retention times are 3.16 min for ABT-578 and 3.65 min for the internal standard. There is a small peak after the main peak for both ABT-578 and the internal standard. These peaks are due to conformational isomers of ABT-578 and of the internal standard [22]. Samples show a consistent ratio between the main peak and the isomer peak. The isomer peak cannot be consistently integrated at low concentration levels, therefore only the main peak was used for quantitation.

3.3. Quantitation method

The peak areas of ABT-578 and internal standard were determined using the SCIEX MacQuan™ software (version 1.6). For each analytical batch, a calibration curve was derived from the peak area ratios (analyte/internal standard) using weighted linear least squares regression of the area ratio versus the concentration of the standards. A 1/concentration^2 weighting was used for the fitting. The regression equation for the calibration curve was used to back-calculate the measured concentration at each standard level and the results

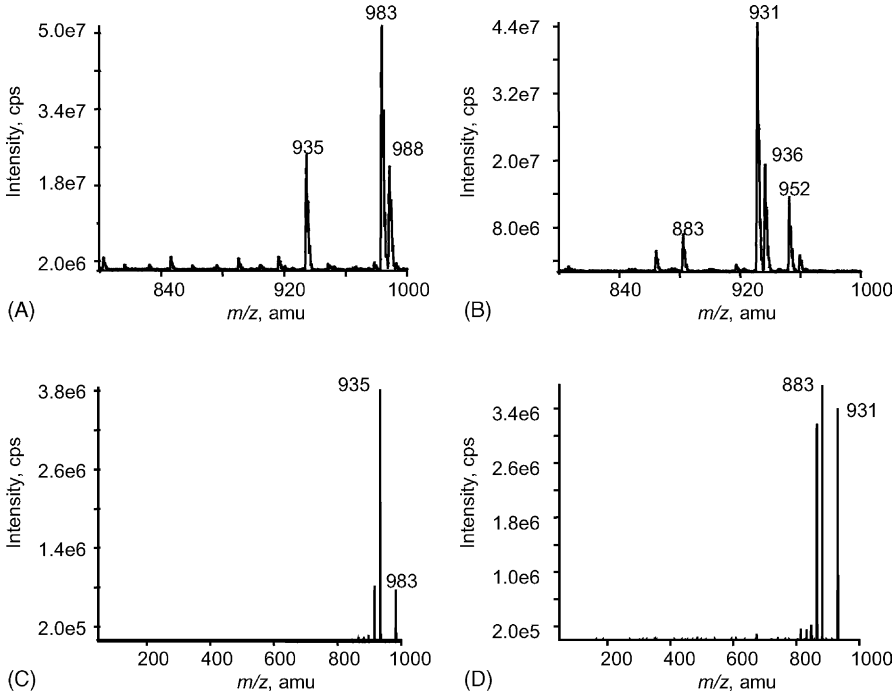

Fig. 2. MS and MS/MS spectra of ABT-578 and internal standard (IS). (A) Mass spectrum of ABT-578; (B) mass spectrum of IS; (C) MS/MS spectrum of ABT−578 and (D) MS/MS spectrum of IS.

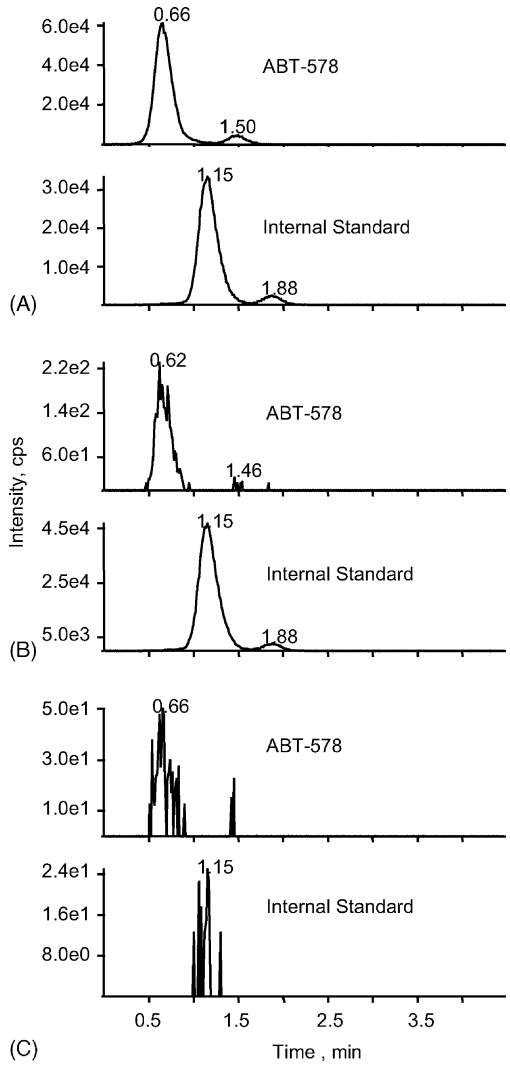

Fig. 3. SRM ion chromatograms of high standard, low standard, and blank. (A) High standard, 200.75 ng/ml ABT-578, 1 μg/ml IS; (B) low standard, 0.20 ng/ml ABT-578, 1 μg/ml IS and (C) extracted blank.

were compared to the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard level measured. Concentrations of the QC samples were also calculated from the regression equation using the observed area ratio for each QC sample. The accuracy of the QC samples was determined by comparing the calculated value to the theoretical value with the result expressed as a percentage of the theoretical value.

Table 2
Summary of extraction recovery for ABT-578

	Concentrations (ng/ml)		Recovery (%)
	Theoretical	Calculated	
Recovery concentration level 1	119.92	145.33	82.5
	119.92	194.96	61.5
	119.92	145.53	82.4
Mean		161.94	75.5
Recovery concentration level 2	3.43	7.52	45.5
	3.43	5.29	64.8
	3.43	4.97	68.9
Mean		5.93	59.7
Recovery concentration level 3	0.41	0.64	63.6
	0.41	0.55	74.5
	0.41	0.63	65.6
Mean		0.61	67.9

3.4. Linearity, recovery and LLOQ

The evaluation of the linearity of the calibration curve was obtained from three batches prepared consecutively. The linear dynamic range evaluated was from 0.20 to 200.75 ng/ml. The correlation coefficient (r) was between 0.9959 and 0.9971. The mean back-calculated concentrations of the standards were between 86.6 and 108.0% of the theoretical concentration (Table 1).

To determine the extraction recovery of ABT-578, three levels of known concentration spiking solutions prepared in the reconstitution solvent were added to the extracted matrix of internal standard and NHB-EDTA. The calculated concentration for each of these samples was obtained using the standard calibration curve. The recovery was calculated by dividing the theoretical concentration by the calculated concentration. Mean extraction recovery was between 59.7 and 75.5% (Table 2). Total procedural recovery is sufficient to achieve accurate, precise and reproducible results at the LLOQ.

Table 1
Statistical summary of calibration curves for ABT-578 standards

	Standard level									Slope	Intercept	r
	1	2	3	4	5	6	7	8	9			
Theoretical concentrations (ng/ml)	0.20	0.50	1.00	2.01	8.03	12.05	20.08	60.23	200.75			
Calculated concentrations (ng/ml)	0.20	0.46	1.02	2.11	8.56	12.86	19.24	46.41	179.76	0.0081	0.0008	0.9971
	0.19	0.53	1.10	2.01	8.61	13.18	18.58	55.04	178.37	0.0096	0.0009	0.9959
	0.19	0.51	1.06	2.16	8.38	12.96	19.28	54.94	176.84	0.0097	0.0008	0.9968
Mean	0.20	0.50	1.06	2.09	8.52	13.00	19.03	52.13	178.32	0.0092	0.0008	0.9966
CV (%)	3.3	6.8	3.6	3.5	1.4	1.3	2.1	9.5	0.8	9.8	8.4	0.1
Theoretical (%)	98.3	99.7	105.8	104.3	106.1	108.0	94.8	86.6	88.8			

Table 3
Statistical summary of the LLOQ measurements for ABT-578

Calculated concentrations (ng/ml) and theory (%)		
Batch ID	LLOQ 0.20	Theory (%)
07/13/01_02	0.14	69.1
07/13/01_02	0.18	88.5
07/13/01_02	0.18	88.0
07/13/01_02	0.17	87.1
07/13/01_02	0.19	94.3
07/13/01_02	0.18	91.0
07/17/01_02	0.21	104.2
07/17/01_02	0.18	91.4
07/17/01_02	0.25	122.2
07/17/01_02	0.19	96.0
07/17/01_02	0.25	122.3
07/17/01_02	0.21	105.6
07/18/01_02	0.21	106.0
07/18/01_02	0.24	120.6
07/18/01_02	0.26	130.4
07/18/01_02	0.22	111.9
07/18/01_02	0.22	108.0
07/18/01_02	0.19	95.8
Mean	0.20	101.8
CV (%)	15.4	
n	18	

Eighteen LLOQ samples (0.20 ng/ml) from three separate batches were used to evaluate the precision and accuracy at the LLOQ. The CV (%) was 15.4% and the mean analytical recovery, expressed as a percent of the theoretical concentration, was 101.8% (Table 3).

3.5. Accuracy and precision

Eighteen replicates of the QC samples from three separate batches were used to evaluate the precision and accuracy at each concentration level. The inter-assay mean accuracy was between 86.4 and 102.5% of the theoretical concentrations. The intra-assay CV (%) was between 1.9 and 13.5% and inter-assay CV (%) was between 4.7 and 11.3% (Table 4).

3.6. Matrix effect and selectivity

Due to the nature of electrospray ionization, matrix components eluted from the HPLC column into the mass spectrometer at the same time as the analyte and/or internal standard may affect the ionization of the compounds of interest. This effect may be seen as either a suppression or enhancement of the analyte and/or internal standard signal, even if the matrix component is not present in the SRM channel monitored for the analytes or internal standards. This matrix effect was evaluated using the method reported previously [23]. A solution containing the analyte and internal standard, approximately 100 ng/ml each in 50/50 (v/v) acetonitrile/water, was infused via a tee connection between the LC column and the mass spectrometer inlet.

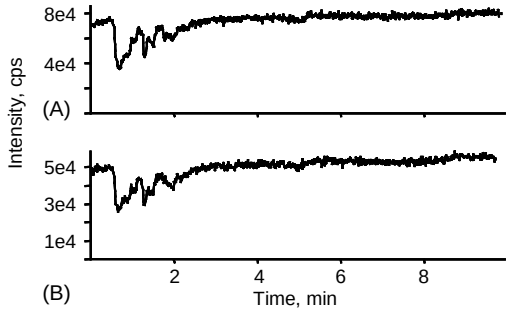

Fig. 4. SRM ion chromatograms during matrix effect evaluation. (A) ABT-578 and (B) IS.

The LC-MS/MS chromatogram (runtime was set to 10 min for each run) after five injections is shown in Fig. 4, the signal intensity of the both analyte and internal standard decreased significantly due to ionization suppression at the solvent front. However, analyte and internal standard signal intensity returned to normal 2.5 min after sample injection.

In addition to matrix ionization effects, the effect of the variation in the individual blood matrix on the accurate measurement of ABT-578 concentration was evaluated. Cynomolgus monkey blood was used as an extreme case for this evaluation. QC samples were prepared in human blood and cynomolgus monkey blood using the same stock solution and were evaluated in the same run. The difference between samples of the same nominal concentration was between −5.9 and 2.8% (Table 5).

Selectivity was evaluated by extracting blank blood samples from six different lots and comparing the response at the retention time of ABT-578 to the response at the LLOQ. As shown in Fig. 3, the response at the retention time of ABT-578 for a blank sample was ∼50 counts per second (cps) while a response of ∼200 cps was obtained for an LLOQ sample.

3.7. Stability

The stability of samples subjected to multiple freeze/thaw cycles with corresponding storage at room temperature was evaluated by subjecting stability QC samples to conditions that simulated repeat sample analysis. The freeze/thaw stability QC samples were assayed along with standards and control QCs that had undergone only one freeze/thaw cycle. The stability QC samples were exposed to room temperature for 33.5 h between 6 freeze/thaw cycles. Comparison of the means of the concentrations show differences between −7.7 and 1.2% (Table 6).

The frozen storage stability of whole blood samples was evaluated as follows. Multiple sets of QC samples were prepared as stability samples for long term stability testing. A set of calibration standards was used to evaluate the stability samples on day one. After a documented period of time

Table 4
Precision and accuracy evaluations for ABT-578 QC samples

Calculated concentrations (ng/ml) and theory (%)								
Batch ID	QC4 158.26	Theory (%)	QC3 4.75	Theory (%)	QC2 2.37	Theory (%)	QC1 0.55	Theory (%)
07/13/01_02	129.53	81.8	5.60	117.9	2.48	104.4	0.47	84.4
	131.80	83.3	4.37	92.1	2.05	86.5	0.47	84.4
	133.15	84.1	4.18	88.0	2.22	93.7	0.43	77.8
	149.30	94.3	5.62	118.3	2.14	90.0	0.51	91.2
	148.33	93.7	4.35	91.6	2.30	97.1	0.50	89.8
	142.77	90.2	4.66	98.2	2.16	90.9	0.47	84.1
Intra-assay								
Mean	139.15	87.9	4.80	101.0	2.23	93.8	0.47	85.3
CV (%)	6.3		13.5		6.7		5.6	
07/17/01_02	130.47	82.4	5.80	122.2	2.25	94.8	0.51	92.6
	136.45	86.2	4.56	96.1	2.22	93.6	0.53	96.6
	142.40	90.0	4.43	93.3	2.32	97.7	0.49	87.9
	136.70	86.4	5.63	118.6	2.18	92.0	0.51	91.8
	146.04	92.3	4.47	94.1	2.22	93.6	0.53	96.1
	134.35	84.9	4.57	96.3	2.13	89.8	0.49	88.2
Intra-assay								
Mean	137.73	87.0	4.91	103.4	2.22	93.6	0.51	92.2
CV (%)	4.1		12.8		2.9		4.0	
07/18/01_02	137.50	86.9	5.44	114.5	2.37	99.7	0.51	92.7
	135.12	85.4	4.75	100.0	2.24	94.2	0.53	95.2
	135.84	85.8	4.67	98.4	2.27	95.6	0.54	97.7
	132.77	83.9	5.48	115.4	1.98	83.5	0.54	96.7
	129.83	82.0	4.48	94.4	2.23	93.9	0.52	94.6
	130.25	82.3	4.51	94.9	2.20	92.5	0.54	96.9
Intra-assay								
Mean	133.55	84.4	4.89	102.9	2.21	93.2	0.53	95.6
CV (%)	2.3		9.3		5.8		1.9	
Inter-assay								
Mean	136.81	86.4	4.86	102.5	2.22	93.5	0.50	91.0
S.D.	6.37		0.55		0.11		0.03	
CV (%)	4.7		11.3		5.1		6.2	
n	18		18		18		18	

Table 5
Matrix effect evaluation for ABT-578 QC samples

	Theoretical concentrations (ng/ml)				
	1.22	4.08	30.61	142.83	346.87
Human					
Mean	1.29	4.26	32.17	148.59	365.65
CV (%)	3.9	6.1	5.0	3.3	1.6
Theoretical (%)	105.4	104.4	105.1	104.0	105.4
n	3	3	3	3	3
Monkey					
Mean	1.24	4.38	32.90	143.70	344.13
CV (%)	4.0	3.0	2.5	3.7	3.1
Theoretical (%)	101.0	107.2	107.5	100.6	99.2
n	6	6	6	6	6
Difference (%)	−3.9	2.8	2.7	−3.3	−5.9

in frozen storage, stability samples were evaluated using a newly prepared set of calibration standards. A set of newly prepared QC samples was used to support the calibration curve. Comparison of the means of the concentrations show differences between −6.4 and 0.7% with CV (%) ≤2.0% (Table 7).

4. Assay application for supporting clinic studies

This assay has been used for the analysis of samples from clinical studies. Selectivity of the method is further demonstrated by the absence of an ABT-578 response above the LLOQ for all pre-dose and placebo samples. Example

Table 6
Freeze/thaw stability evaluation for ABT-578 QC samples

	Theoretical concentrations (ng/ml)	
	0.50	159.75
1 freeze/thaw cycle		
Mean	0.52	158.26
CV (%)	5.8	0.6
Theoretical (%)	104.2	99.1
n	3	3
6 freeze/thaw cycles		
Mean	0.48	160.09
CV (%)	2.1	1.6
Difference (%)	−7.7	1.2
n	3	3

Table 7
Frozen storage stability evaluation for ABT-578 QC samples

	Theoretical concentrations (ng/ml)	
	0.50	160.08
Day 1	0.50	165.41
Mean		
CV (%)	4.0	2.0
Theoretical (%)	100.0	96.7
n	6	6
Day 112	0.50	154.87
Mean		
CV (%)	2.0	2.0
Difference (%)	0.7	−6.4
n	3	3

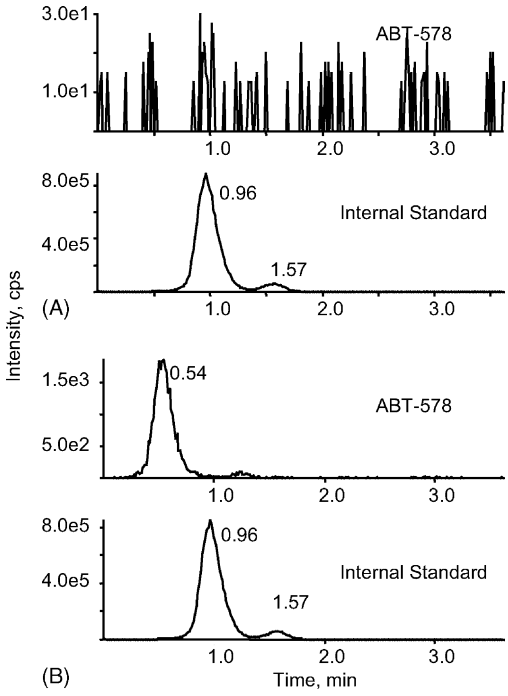

Fig. 5. Example of the SRM ion chromatogram of a patient sample. (A) Pre-dose sample and (B) 72 h sample after a bolus dose.

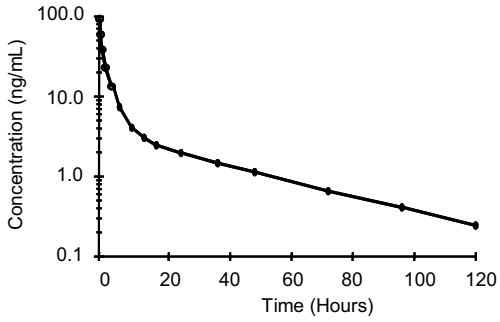

Fig. 6. Example of a patient concentration profile following a bolus dosing.

chromatograms from study samples are shown in Fig. 5. The concentration profile of a patient is shown in Fig. 6. The dynamic range of the assay is adequate to meet the requirement of clinical sample analysis.

5. Conclusions

We have reported methodology and validation of the bioanalytical assay for the measurement of ABT-578 in human whole blood. Utilization of a semi-automated 96-well format liquid–liquid extraction method for sample preparation simplified and accelerated the sample preparation process. Use of an LC-MS/MS system for separation and detection, combined with pre-column regeneration, allowed for the validation of an assay with a 1000 fold linear dynamic range with good accuracy and precision.

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