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Simultaneous determination of ABT-888, a poly (ADP-ribose) polymerase inhibitor, and its metabolite in human plasma by liquid chromatography/tandem mass spectrometry

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Abstract

A reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was developed and validated for simultaneous determination of ABT-888 and its major metabolite (M8) in human plasma. Sample preparation involved a liquid-liquid extraction by the addition of 0.25 ml of plasma with 10 μ l of 1M NaOH and 1.0 ml ethyl acetate containing 50 ng/ml of the internal standard zileuton. The analytes were separated on a Waters XBridge C₁₈ column using a gradient mobile phase consisting of methanol/water containing 0.45% formic acid at the flow rate of 0.2 ml/min. The analytes were monitored by tandem-mass spectrometry with electrospray positive ionization. Linear calibration curves were generated over the ABT-888 and M8 concentration ranges of 1-2000 ng/ml in human plasma. The lower limits of quantitation (LLOQ) were 1 ng/ml for both ABT-888 and M8 in human plasma. The accuracy and within- and between-day precisions were within the generally accepted criteria for bioanalytical method (<15%). This method was successfully employed to characterize the plasma concentration-time profile of ABT-888 after its oral administration in cancer patients.

Keywords

ABT-888; PARP inhibitor; High performance liquid chromatography; Mass Spectrometry; LC-MS/MS; Pharmacokinetics

1. Introduction

ABT-888 is an orally available, small molecule inhibitor of poly (ADP-ribose) polymerase (PARP)-1 and PARP-2. PARP-1 and PARP-2 are nuclear enzymes that are involved in DNA repair via poly (ADP-ribosyl)ation of histones and DNA repair enzymes. They serve as DNA damage sensors and signaling molecules for DNA repair. PARP activity is essential for the repair of single-stranded DNA (ssDNA) break through the base excision repair pathway [1,2], and elevated PARP levels in tumor cells can result in resistance to cytotoxic chemotherapy and radiation therapy [3,4]. Thus, inhibition of PARP activity may sensitize tumor cells to chemotherapy and radiation therapy. ABT-888 has been demonstrated to potentiate a variety of DNA-damaging agents (e.g., cisplatin, carboplatin, cyclophosphamide, irinotecan, and temozolomide) and radiation in various xenografts/syngeneic tumor models [5-7]. ABT-888 has been evaluated in a phase 0 clinical trial, in which the drug was administered as a single oral dose of 10, 25, or 50 mg in the patients with advanced malignancies. ABT-888 was well tolerated, and produced statistical

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significant inhibition of poly (ADP-ribose) levels in tumor biopsy and peripheral blood mononuclear cells at the 25-mg and 50-mg dose levels [8]. Currently, ABT-888 is being evaluated in a phase I trial in combination with irinotecan in patients with advanced or refractory solid tumors at several institutions including the Karmanos Cancer Institute at Wayne State University (HIC# 090107M1F).

To characterize the pharmacokinetics of ABT-888 in cancer patients, a specific, sensitive, accurate, and reproducible method for quantitation of ABT-888 and its major metabolite was critically needed. A liquid chromatography coupled with single quadrupole mass spectrometry (LC-MS) method for the quantitation of ABT-888 and its major metabolite (M8) in human plasma has been published [9]. In this published method, ABT-888 and M8 were extracted from 0.2 ml of plasma sample; the lower limit of quantitation (LLOQ) was determined at 10 ng/ml for both ABT-888 and M8; the linear calibration curves were generated over the ABT-888 and M8 concentration range of 10 – 1000 ng/ml in human plasma; the total running time for each sample was 25 min. In this report, we developed and validated a high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for determination of ABT-888 and M8 in human plasma. The present method demonstrated an improved sensitivity, with the LLOQ being achieved at 1 ng/ml for both ABT-888 and M8. This method also generated a wider linear calibration curve range over the ABT-888 and M8 concentration ranging from 1 to 2000 ng/ml in human plasma. In addition, the present method is faster, with the total running time of 10 min for each sample.

2. Experimental

2.1. Chemicals and reagents

ABT-888 (A-861695) and its major metabolite M8 (A-925088) were generously provided by Abbott Laboratory (Abbott Park, IL, USA). The internal standard, zileuton [N-(1-benzobthien-2-ylethyl)-N-hydroxyurea] was obtained from Rhodia Pharma Solutions Ltd (Northumberland, UK). All other chemicals and reagents were HPLC grade. Water was filtered and deionized with a US Filter PureLab Plus UV/UF system (Siemens, Detroit, MI, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from six different healthy donors was obtained from Innovative Research Inc. (Novi, MI, USA).

2.2. Stock solutions and standards

Stock solutions of ABT-888, M8, and zileuton (internal standard) were prepared in methanol at a concentration of 1 mg/ml, and stored in glass vials at – 20°C. Working stock solutions were prepared fresh on each day of analysis as serial dilutions in methanol. The calibration curves were constructed by simultaneously spiking ABT-888 and M8 in blank plasma at the concentrations of 1, 5, 10, 20, 50, 100, 200, 500, 1000 and 2000ng/ml. Quality control (QC) samples were prepared in blank plasma at ABT-888 and M8 concentrations of 1 (LLOQ), 15, 800 and 1600ng/ml. All standards and QC samples were prepared fresh daily. For long-term and freeze-thaw stability, QC samples were prepared as a batch and stored at –80°C.

2.3. Sample preparation

Prior to extraction, frozen samples were thawed in a water bath at ambient temperature. A 250 µl aliquot of plasma was added to a 1.5ml polypropylene eppendorf tube followed by spiking with 10µl of 1M NaOH and 1ml of ethyl acetate containing 50ng/ml of internal standard, zileuton. The mixture was vortex-mixed for approximately 1 minute, and centrifuged at 14,000 rpm for 5 minutes at ambient temperature. The top layer was transferred to a 1.5 ml polypropylene screw top tube, and evaporated to dryness under a stream of nitrogen in a water bath at 50 ± 5°C. An aliquot of 100 µL of reconstitution

solution (methanol/0.45% formic acid in water, 20:80, v/v/) was added into the tube. The mixture was vortex-mixed for 30 seconds, and centrifuged at 14,000rpm for 5 minutes at 4°C. The supernatant was transferred into a 250 µl polypropylene autosampler vial, sealed with a Teflon crimp cap. A volume of 10 µl was injected into the HPLC instrument using a temperature-controlled autosampling device (set at 4°C).

2.4. Chromatographic and mass-spectrometric conditions

Chromatographic analysis was performed using a Waters Model 2695 separations system (Milford, MA, USA). Separation of the analytes from potentially interfering material was achieved at 30°C using Waters XBridge C₁₈ column (50 × 2.1 mm i.d.) packed with a 3.5 µm C₁₈ stationary phase, protected by a Waters XBridge guard column (10 × 2.1 mm i.d.) packed with 3.5 µm C₁₈ material (Milford, MA, USA). The mobile phase used for the chromatographic separation was composed of methanol (A) and 0.45% formic acid in water (B), and was delivered at a flow rate of 0.2 ml/min using a gradient elution with 20% of A for 0.5 min and then the proportion of A increasing from 20% to 90% in 1 min, staying at 90% for 4.5 min, then decreasing to 20% in 1min, keeping at 20% for additional 3 min. The column effluent was monitored using a Waters Quattro Micro™ triple quadrupole mass-spectrometric detector (Milford, MA, USA). The instrument was equipped with an electrospray ionization source, and controlled by the Masslynx version 4.1 software, running under Windows XP 2000 on an IBM IntelliStation computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 15 V for ABT-888, 25 V for M8, and 13 V for internal standard zileuton. Samples were introduced into the ionization source through a heated nebulized probe (350°C). The spectrometer was programmed to allow the [MH]⁺ ion of ABT-888 at *m/z* 245.2, M8 at *m/z* 259.1, and zileuton at *m/z* 237.1 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 9, 15, and 9 eV for ABT-888, M8, and zileuton, respectively. The product ions for ABT-888 (*m/z* 83.5), M8 (*m/z*, 241.9) and zileuton (*m/z* 160.8) were monitored through the third quadrupole (Q3). Argon was used as collision gas at a pressure of 0.00172 mBar, and the dwell time per channel was 0.5 sec for data collection.

2.5. Method validation

2.5.1. Specificity—The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from 6 different donors for the presence of endogenous or exogenous interfering peaks. The plasma samples without (blank) and with spiking the analytes (ABT-888 and M8 at the LLOQ) and the internal standard (zileuton at 50 ng/ml) were prepared and extracted. The interfering peak area should not exceed 20% of the analyte peak area at the LLOQ and 5% of the internal standard peak area.

2.5.2. Calibration curve—Linearity was assessed at the analyte (ABT-888 or M8) concentration ranging from 1 to 2000 ng/ml. Calibration curves were built by fitting the analyte concentrations of the calibrators versus peak area ratios of the analyte to internal standard using least-squares non-linear regression analysis with different weighting scheme (i.e., 1, 1/x, and 1/x²). The selection of weighting scheme was guided by evaluation of goodness-of-fit criteria including correlation coefficient (*R*²), % recovery of back-calculated calibrators and QCs, and residual plots.

2.5.3. Accuracy and precision—Validation runs for the calibrator standards (in duplicate) and QCs (in quintuplicate) including LLOQ, low, medium, and high were performed on four days. The accuracy was assessed as the relative percentage of the back-calculated concentration to nominal concentration, which was equal to determined concentration/nominal concentration × 100%. The within- and between-day precisions were

estimated by one-way analysis of variance (ANOVA) using the JMPTM statistical discovery software version 5 (SAS Institute, Cary, NC, USA). The between-day variance (VAR_{bet}), the within-day variance (VAR_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The within-day precision (WDP) was calculated as:

$$WDP=100 \times \left(\sqrt{VAR_{wit}}/GM \right)$$

The between-day precision (BDP) was defined as:

$$BDP=100 \times \left(\sqrt{((VAR_{bet} - VAR_{wit})/n)/GM} \right)$$

where n represents the number of replicate observations within each day.

2.5.4. Matrix effect and extraction recovery—Matrix effect and extraction recovery were assessed in 6 different sources of human plasma, as described previously [10,11]. Three sets of QC samples (including low, medium, and high concentrations of ABT-888 and M8) were prepared. The first set (set 1) of QC samples was prepared in mobile phase to evaluate the detector response for neat standards of the analytes (i.e., ABT-888 and M8) and internal standard (i.e., zileuton). The second set (set 2) of QC samples was prepared in plasma extracts from 6 different sources of plasma and spiked after extraction. The third set (set 3) QC samples was prepared in plasma from the same 6 different sources as in set 2, but the analytes were spiked in plasma before extraction. The matrix effect is expressed as the ratio of the mean peak area of an analyte spiked postextraction (set 2) to the mean peak area of the same analyte standard (set 1). The extraction recovery is calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked postextraction (set 2).

The low, medium, and high working standard solutions containing 0.15, 16, and 32 $\mu\text{g/ml}$ of ABT-888 and M8 were prepared in methanol. The stock solution of internal standard was prepared in methanol at the zileuton concentration of 0.1 $\mu\text{g/ml}$. In set 1, the QC samples were prepared by mixing 100 μl of the low, medium, or high working standards, 100 μl of internal standard stock solution, and 100 μl of the mobile phase (total volume 300 μl). After vortex-mixing, 10 μl was injected directly into the LC-MS/MS system. In set 2, 1 ml of plasma from 6 different donors was spiked with 200 μl of methanol (to simulate the addition of 100 μl of working standard and 100 μl of internal standard stock solution into plasma in set 3). After vortex-mixing, the plasma was basified with 40 μl of 1M NaOH and extracted with 4 ml of ethyl acetate, as described in section 2.3. The residue was reconstituted in 300 μl of mobile phase, and 100 μl of the extraction solution was transferred into a 1.5-ml eppendorf tube followed by spiking with 100 μl of the low, medium, or high working standards and 100 μl of internal standard stock solution (total volume 300 μl). After vortex-mixing, 10 μl was injected into the LC-MS/MS system. In set 3, the QC samples were prepared by spiking 100 μl of the low, medium, or high working standards and 100 μl of internal standard stock solution into 1 ml plasma from the same different donor as set 2. The plasma QC samples were basified and extracted in the same manner as in set 2. The residue was reconstituted in 300 μl of mobile phase, and 10 μl was injected into the LC-MS/MS system.

2.5.5. Stability—The short-term (bench-top) stability of the analytes (ABT-888 and M8) in methanol (working solution) at the concentration of 100 and 1 $\mu\text{g/ml}$ as well as in plasma

at the concentrations of 15 and 1600 ng/ml were tested at ambient temperature (25°C) for 4 h. The autosampler stability of the analytes (ABT-888 and M8) in the reconstitution solution (methanol/0.45% formic acid in water, 20:80, v/v) was examined at 4°C for 12 h after the low and high QC plasma samples (at the concentration of 15 and 1600 ng/ml) were processed. The freeze-thaw stability of the ABT-888 and M8 in plasma was assessed at the low and High QC concentrations of 15 and 1600 ng/ml through three freeze-thawing cycles. The long-term stability of ABT-888 and M8 in stock solution (1 mg/ml) and in plasma (at 15 and 1600 ng/ml) was investigated up to 12 months and 8 months, respectively. All QCs were run in triplicate.

2.6. Pharmacokinetic analysis

ABT-888 is currently being evaluated in a Phase I clinical trial in combination with irinotecan in patients with advanced or refractory solid tumors. ABT-888 was administered orally twice daily from day 1 through day 14, and irinotecan was administered by 90-min intravenous infusion (at the dose of 100 and 125 mg/m²) on days 1 and 8. One treatment cycle included 21 days. The first ABT-888 dose level of 10 mg twice daily has been evaluated in five patients. The dose escalation is continued. The protocol was approved by the Institutional Review Board of the participated institutions. All patients provided a written informed consent.

To examine the pharmacokinetics of ABT-888, blood samples were collected in heparinized tubes on day -1 (ABT-888 alone) and day 8 (ABT-888 in combination with irinotecan) in cycle 2 at the following time points: pretreatment, 30 min (after ABT-888 dosing), 1, 1.5, 3.5, 5.5, 8.5, 10 and 28 hours. The blood samples were immediately placed in an ice bath and then centrifuged at 1500 g at 4°C for 10 minutes. Plasma was separated and split into two aliquots, and stored at -80°C until analysis.

The concentrations of ABT-888 and M8 in patient plasma samples were determined using the described validated method. The pharmacokinetic parameters including the maximum plasma concentration ($C_{\max}$), time to reach the $C_{\max}$ ($T_{\max}$), area under the concentration-time curve to the last sampling time point (AUC_{last}) for ABT-888 and M8 in individual patients were estimated using noncompartmental analysis with the computer software program WinNonlin version 5.0 (Pharsight Corporation, Mountain View, California).

3. Results and discussion

3.1. Detection and chromatography

The mass spectrum of ABT-888 and M8 showed the protonated molecules ($[MH^+]$) at m/z 245.2 and 259.1, respectively. The collision energy fragmented the analytes into several fragments. The major fragments observed were at m/z 83.5 and 241.9 and were selected for subsequent monitoring in the third quadrupole for ABT-888 (Fig. 1a) and M8 (Fig. 1b), respectively. The internal standard, zileuton, had protonated molecules ($[MH^+]$) at m/z 237.1 and produced a major fragment at m/z 160.8 (Fig. 1c).

Representative chromatograms of blank and spiked human plasma samples as well as a patient sample collected at 3.5 h after oral administration of a single dose of ABT-888 (5 mg) that were monitored at m/z 245.2 → 83.5 (for ABT-888) (Fig. 2a, 2d, and 2g), m/z 259.1 → 241.9 (for M8) (Fig. 2b, 2e, and 2h), and m/z 237.1 → 160.8 (for zileuton) (Fig. 2c, 2f, and 2i) are shown in Fig. 2. The mean ($\pm$ standard deviation) retention times for ABT-888, M8, and zileuton under the optimal conditions were at 1.25 ± 0.01 , 1.30 ± 0.02 , and 6.48 ± 0.02 min, respectively, with an overall chromatographic run time of 10 minutes (Fig. 2). The selectivity for the analysis was shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention times of the analytes

and internal standard in human plasma from six different donors (Fig. 2). During implementation of this assay, pre-treatment plasma samples from the cancer patients were analyzed with this assay with no interferences noted.

3.2. Calibration Curves

The calibration curves were established over the nominal concentration range of 1 to 2000 ng/ml for both ABT-888 and M8. The relationship between peak area ratios of the analyte to the internal standard versus the analyte concentrations was best fitted by a linear equation, expressed as $y = a \cdot x + b$, where y is peak area ratio, x is the analyte concentration, a and b are fitted parameters. A weighting factor, which is inversely proportional to the variance at the given concentration level (x^2), was used. This weighting factor was chosen compared to uniform weighting and weighting by $1/x$ because the weighting factor of $1/x^2$ produced the best goodness-of-fit in terms of the R^2 value, intercept closest to a zero value, percent recovery of calibrators and QCs, and distribution of residues. For both ABT-888 and M8 curves, a mean least-squares linear regression correlation coefficient (R^2) of > 0.99 was obtained in all analytical runs. The distribution of residuals was random, normally distributed, and centered on zero (data not shown). For each calibrator standard (in duplicate each day for 3 days, $n = 6$) on the calibration curves of ABT-888 and M8, the average accuracy in terms of percent recovery of the back-calculated relative to nominal concentration ranged from 94.2% to 103.1% ($n = 6$) and 97.3% to 102.7% ($n = 6$), respectively; the within- and between-day precisions (expressed as the relative standard deviations) were less than 14.3% and 6.8% for all calibrator standards of ABT-888 and M8, respectively (Table 1).

3.3. Accuracy and precision

The LLOQ for both ABT-888 and M8 was established at 1 ng/ml, at which the mean signal-to-noise ratio was 70 and 23 from 20 observations, respectively. For the QC samples prepared by spiking human plasma with ABT-888 at the concentrations of 1 (LLOQ), 15, 800 and 1600 ng/ml, the average accuracy (expressed as the percent recovery of the back-calculated relative to nominal concentration) ranged from 98.3% to 113.6%; the within- and between-day precisions were all less than 11.8% (Table 2). For the QCs of M8 at the concentrations of 15, 800, 1600 ng/ml, the average accuracy ranged from 88.7% to 109.5%, and within- and between-day precisions (expressed as the relative standard deviations) were less than 8.4% (Table 2).

3.4. Matrix effect and extraction recovery

The matrix effect was examined in 6 different sources of human plasma to assess the possibility of ionization suppression or enhancement for ABT-888, M8, and the internal standard zileuton. At the concentrations of 15, 800, and 1600 ng/ml, the average matrix effect (factor) from six different sources of plasma was determined as 99.4%, 99.9%, and 103.4%, respectively, for ABT-888; and the average matrix effect was 88.9%, 87.8%, and 94.0%, respectively, for M8 (Table 3). The average matrix effect for the internal standard zileuton was determined as 95.8% at the concentration of 10 ng/ml from six different source of human plasma (Table 3). The variability in matrix effect, as measured by the coefficient of variation (CV%) from the 6 different sources of plasma, was less than 7% for ABT-888, M8, and zileuton (Table 3). These results suggest that there is no apparent ionization suppression or enhancement from the matrix (human plasma) for the analytes (i.e., ABT-888, M8, and the internal standard zileuton).

The extraction recovery is determined as the ratio of the peak area of an analyte from an extracted sample (set 3) to the peak area of the analyte from an unextracted sample (set 2) containing the same amount of analyte that was added to the extracted sample. The

extraction recovery need not be very high, but it should be consistent and reproducible [11]. At the concentrations of 15, 800 and 1600 ng/ml, the average extraction recovery of ABT-888 and M8 from 6 different sources of plasma ranged from 27.7% to 34.3% and from 15.1% to 19.4%, respectively (Table 3). The average extraction recovery for zileuton was determined as 57.4% (Table 3). The variability in extraction recovery, as measured by the CV% from the 6 different sources of plasma, was within 15% for all the analytes (Table 3). These results suggest that the extraction recovery was consistent and reproducible for both the analytes (ABT-888 and M8) and internal standard (zileuton).

3.5. Stability

The short- and long-term stability of ABT-888 and M8 was demonstrated in Table 4. At ambient temperature (~25°C), both ABT-888 and M8 were stable for at least 4 h in methanol working solution at the concentrations of 1 and 100 µg/ml. In plasma samples at the concentrations of 15 and 1600 ng/ml, both ABT-888 and M8 were stable for at least 4 h. In the autosampler (set at 4°C), ABT-888 and M8 were stable for at least 12 h in the reconstitution solution (methanol/0.45% formic acid in water, 20:80, v/v), allowing the assay to be performed continuously overnight for a large number of samples (Table 4). Freeze-thaw stability, which was assessed at ABT-888 or M8 concentration of 15 and 1600 ng/ml, showed no significant (<14.1% for ABT-888 and < 9.7% for M8) degradation through three full cycles of freeze-thaws. The long-term stability tests suggested that ABT-888 and M8 were stable in methanol (stock solution, 1 mg/ml) at -20°C for at least 12 months (with degradation less than 10%). They were stable in human plasma at -80°C for at least eight month (with degradation less than 15%).

3.6. Plasma concentration–time profile

This LC-MS/MS method was successfully employed to study the pharmacokinetics of ABT-888 after its oral administration twice daily in cancer patients in a dose-escalation phase I trial. The pharmacokinetic profile of ABT-888 was characterized in the first 5 patients who were treated with ABT-888 at the dose of 10 mg twice daily. On day -1 in cycle 1, following a single oral dose of 5 mg (half of the total daily dose), ABT-888 achieved a mean C_{max} of 22 ng/ml (ranging from 10 to 31 ng/ml) at a mean T_{max} of 2.4 h (ranging from 1.5 h to 3.6 h); M8 achieved a mean C_{max} of 11 ng/ml (ranging from 7 to 14 ng/ml) at a mean T_{max} of 5.2 h (ranging from 3.5 to 9.8 h). The ratio of AUC_{last} of M8 to ABT-888 ranged from 0.3 to 1.9, with a mean value of 1.0. Fig. 3 shows the representative plasma concentration-time profiles of ABT-888 and M8 in one patient who received single dose of 5 mg ABT-888.

4. Conclusion

In summary, a sensitive and reliable LC-MS/MS method was developed and validated for the determination of ABT-888 and M8 simultaneously in human plasma. The LLOQ for both ABT-888 and M8 was determined at 1 ng/ml in plasma, and the calibration curves for these two compounds were established in the range of 1 to 2000 ng/ml. This method was easily applied for quantitation of ABT-888 and M8 in a large number of plasma samples and allowed characterization of pharmacokinetic profiles of ABT-888 and M8 over a wide dose range in the dose-escalation phase I trial.

Acknowledgments

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Fig. 1. Daughter mass spectrum of ABT-888, M8, and the internal standard zileuton with monitoring at m/z 245.2 $\rightarrow$ 83.5 (a), m/z 259.1 $\rightarrow$ 241.9 (b), and 237.1 $\rightarrow$ 160.8 (c), respectively.

Fig. 2.

Chromatograms of blank plasma (a, b, c), spiked plasma with ABT-888 and M8 at the LLOQ (1 ng/ml) (d, e, f), and a patient sample collected at 3.5 h after oral administration of a single dose of ABT-888 (5 mg) (g, h, i) that was monitored at m/z 245.2 – 83.5 for ABT-888 (a, d, g), m/z 259.1 – 241.9 for M8 (b, e, h), and m/z 237.1 – 160.8 for zileuton (c, f, i). The retention times for ABT-888, M8, and zileuton were 1.25 ± 0.01 , 1.30 ± 0.02 , and 6.48 ± 0.02 min, respectively.

Fig. 3.
Representative plasma concentration-time profiles of ABT-888 and M8 in a cancer patients following oral administration of a single dose of ABT-888 at the dose of 5 mg.

Table 1

Accuracy, within- and between-day precisions of calibrator standards ^a in the calibration curves of ABT-888 and M8

Analyte	Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Average Accuracy (%)	Within-day (%)	Between-day (%)
ABT-888	1 (LLOQ)	1.0 ± 0.1	99.1	10.1	_b
	5	5.0 ± 0.3	100.8	7.6	_b
	10	10.2 ± 0.5	101.5	4.8	1.1
	20	20.2 ± 1.4	101.0	3.0	6.8
	50	50.5 ± 2.8	101.1	6.5	_b
	100	102.9 ± 4.7	102.9	5.6	_b
	200	206.3 ± 9.2	103.1	4.7	_b
	500	503.3 ± 44.8	100.7	8.0	4.3
	1000	1000.2 ± 90.6	100.0	8.1	4.3
	2000	1883.7 ± 104.5	94.2	7.1	_b
M8	1 (LLOQ)	1.0 ± 0.1	98.5	5.4	_b
	5	5.0 ± 0.3	100.3	5.7	_b
	10	10.0 ± 0.7	100.2	8.6	_b
	20	20.1 ± 1.8	100.5	7.8	4.1
	50	49.7 ± 4.7	99.5	7.6	5.9
	100	102.7 ± 7.1	102.7	7.6	_b
	200	201.4 ± 13.2	100.7	7.7	_b
	500	502.9 ± 48.4	100.6	9.2	3.2
	1000	973.1 ± 142.8	97.3	14.3	3.4
	2000	1982.9 ± 149.3	99.1	8.3	_b

^a Each calibrator was evaluated in duplicate on four days.

^b No additional variation was observed as a result of performing assay in different days.

Table 2Accuracy, within- and between-day precision for the QC samples ^a of ABT-888 and M8.

Analyte	Nominal Concentration (ng/ml)	Determined concentration (ng/ml)	Average accuracy (%)	Within-day (%)	Between-day (%)
ABT-888	1 (LLOQ)	0.98 ± 0.14	98.3	9.5	11.8
	15	16.43 ± 0.62	109.6	2.9	2.8
	800	909.21 ± 32.22	113.6	3.6	<i>_b</i>
	1600	1796.20 ± 121.21	112.3	5.6	4.2
M8	1 (LLOQ)	0.89 ± 0.07	88.7	7.8	1.8
	15	15.29 ± 1.03	101.9	5.9	3.5
	800	876.36 ± 50.56	109.5	6.0	<i>_b</i>
	1600	1728.10 ± 145.76	108.0	8.4	1.0

^aPerformed in quintuplicate on four days.^bNo additional variation was observed as a result of performing assay in different days.

Table 3

Matrix effect and extraction recovery for ABT-888, M8, and the internal standard zileuton in 6 different sources of human plasma

Analyte	Nominal concentration (ng/ml) ^a	Mean peak area			Matrix effect (%) ^e	Extraction recovery (%) ^f
		Set 1 ^b	Set 2 ^c	Set 3 ^d		
ABT-888						
	15	3086	3065	849	99.4 (6.8%)	27.7 (13.1%)
	800	139001	138927	42023	99.9 (5.9%)	30.4 (11.4%)
	1600	244859	253232	86813	103.4 (3.4%)	34.3 (6.5%)
M-8						
	15	1181	1049	158	88.9 (5.1%)	15.1 (7.8%)
	800	56963	50035	8764	87.8 (5.4%)	17.7 (8.2%)
	1600	101142	95075	18403	94.0 (2.4%)	19.4 (4.0%)
Zileuton						
	10	1990	1907	1095	95.8 (1.4%)	57.4 (11.1%)

^aValues are shown as the nominal concentrations of the analyte spiked in plasma before extraction (set 3). The same amounts of the analyte as in set 3 were spiked in mobile phase and in plasma extract for set 1 and set 2, respectively.

^bData are shown as the mean peak area of an analyte in neat solution from triplicate measurements.

^cData are shown as the mean peak area of an analyte spiked postextraction in plasma extracts from 6 different sources of human plasma, each source of plasma in triplicate measurements.

^dData are shown as the mean peak area of an analyte spiked before extraction in plasma from the same 6 different sources of human plasma as in set 2, each source of plasma in triplicate measurements.

^eMatrix effect is expressed as the ratio of the mean peak area of an analyte spiked postextraction (set 2) to the mean peak area of the same analyte standard (set 1). Data are shown as the mean (%CV) from six different sources of plasma.

^fExtraction recovery is calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked postextraction (set 2). Data are shown as the mean (%CV) from six different source of plasma.

Table 4Assessment of stability of ABT-888 and M8 ^a

	ABT-888 (ng/ml) 15	1600	M8 (ng/ml) 15	1600
Bench-top stability (in plasma) (25°C) ^b				
1.0 h	100.0	96.6	99.0	97.6
2.0 h	97.8	109.1	98.3	109.8
3.0 h	100.7	100.5	98.1	98.5
4.0 h	108.9	93.6	102.4	90.0
Autosampler stability (in methanol/0.45% formic acid in water, 20:80, v/v) (4°C) ^c				
1.0 h	97.4	105.1	91.7	97.1
4.0 h	102.6	93.5	91.7	95.9
8.0 h	105.1	104.2	91.7	98.1
12.0 h	105.1	106.8	91.7	97.8
Freeze-thaw stability (in plasma) (−80°C) ^b				
Cycle 1	106.2	114.1	98.8	108.4
Cycle 2	106.7	111.1	96.1	108.5
Cycle 3	111.5	113.0	101.7	109.7
Long-term stability (in plasma) (−80°C) ^b				
4 month	94.0	109.4	95.8	105.4
8 month	108.6	113.2	92.4	100.1

^aStability data were expressed as mean percentage of the analyte concentration determined at certain time point relative to that at time zero (%)^bEach concentrations were assessed in triplicate.^cInjected repeatedly for 12 h with one sample.