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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 189–195
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

# Automated 96-well liquid–liquid back extraction liquid chromatography–tandem mass spectrometry method for the determination of ABT-202 in human plasma

Naxing Xu*, Grace E. Kim, Hope Gregg, Azza Wagdy, Brendan A. Swaine, Min S. Chang, Tawakol A. El-Shourbagy

*Abbott Laboratories, Department of Clinical Drug Analysis, Dept. R46W, Bldg. AP13A-2, 100 Abbott Park Road, Abbott Park, IL 60064-6126, USA*

Received 25 January 2004; received in revised form 14 May 2004; accepted 16 May 2004

Available online 24 July 2004

**Abstract**

A high-throughput bioanalytical method using automated sample transferring, automated liquid–liquid back extraction and liquid chromatography–tandem mass spectrometry was developed in a GLP regulated environment for the determination of ABT-202 in human plasma. Samples of 0.30 ml were transferred into 96-well plate using an automatic liquid handler. Automated liquid–liquid extraction (LLE) was carried out on a 96-channel programmable liquid handling workstation using methyl *tert*-butyl ether as the extraction solvent. A dual-HPLC with single mass spectrometer configuration was utilized to provide a reliable and routine means to increase sample throughput. The standard curve range was 0.38–95.02 ng/ml. There was no interference from endogenous components in the blank plasma tested. The accuracy (% bias) at the lower limit of quantitation (LLOQ) was 7.7% and the precision (% CV) for samples at the LLOQ was 4.7%. The inter-day % CV and % bias of the quality control samples were ≤6.8 and ≤7.6%, respectively. Coefficients of determination, a measure of linearity, ranged from 0.994 to 0.997. The method was accurate and reproducible and was successfully applied to generate plasma concentration-time profiles for human subjects after low oral doses of the compound.


*Keywords:* High throughput analysis; Liquid–liquid back extraction; Liquid chromatography; Tandem mass spectrometry; Automated procedure

## 1. Introduction

ABT-202, as shown in Fig. 1, is a novel neuronal nicotinic receptor (NNR) agonist that is being developed by Abbott Laboratories for pain modulation. As a non-opioid analgesic, ABT-202 has shown to be active in pre-clinical studies. In order to further evaluate the pharmacokinetic parameters and exposure of ABT-202, a very sensitive and selective analytical method for the determination of ABT-202 in human plasma is required.

ABT-202 is a relatively polar and volatile compound in its free base form. Previous methods for the analysis of compounds similar to ABT-202 involved fluorescence detection and have yielded sample preparation methods consisting of multiple steps including: organic solvent extraction, chemical derivatization, solvent extraction, evaporation and sample reconstitution steps [1–3]. In recent years, LC/MS/MS has been extensively used in the pharmaceutical industry as a tool for quantitative determination of drug and metabolite in biological matrices. Generally, LC/MS/MS offers direct analysis of a wide spectrum of compounds with great sensitivity and selectivity [4–7]. However, the sample preparation procedure is the most labor-intensive step in an LC/MS/MS assay. To increase sample throughput, robotic liquid handling systems have been developed to automate steps in protein precipitation, LLE or solid phase extraction (SPE) methods in a 96- or 384-well plate [8–10]. On-line sample preparation techniques such as turbulent flow chromatography or the Prospekt system for solid-phase extraction have also been used for high-throughput bioanalysis [11,12].

Because of its analogous structure, A443919 (Fig. 1) was chosen as the internal standard (IS). This paper describes the method and validation for a high throughput LC/MS/MS method for the determination of ABT-202 in normal human

* Correspondence author. Tel.: +1 847 938 8158; fax: +1 847 938 7789.
*E-mail address:* naxing.r.xu@abbott.com (N. Xu).

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doi:10.1016/j.jpba.2004.05.013

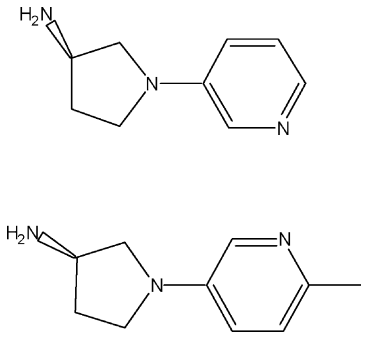

Fig. 1. The chemical structure of ABT-202 (top) and the internal standard, A443919 (bottom).

plasma. The method described here utilized automated sample transferring and LLE in 96-well plate format. Particular effort was made to automate the sample preparation step and increase sample throughput of the analysis.

## 2. Experimental

### 2.1. Chemicals

Methanol, methyl *tert*-butyl ester (MTBE), trifluoroacetic acid (TFA), and formic acid, HPLC grade, were purchased from EM Science (Gibbstown, NJ, USA). Ammonium acetate and sodium carbonate, ACS grade, was purchased from J. T. Baker (Phillipsburg, NJ, USA). Water was produced by a Millipore (Bedford, MA, USA) Milli-Q unit. Nicotine was purchased from Sigma-Aldrich (Milwaukee, WI, USA). ABT-202 and internal standard A443919 were obtained from Abbott Laboratories (Abbott Park, IL, USA). Normal human plasma with sodium heparin as anticoagulant was purchased from Biological Specialties Corporation (Colmar, PA, USA).

### 2.2. Standard and quality control (QC) solutions

Stock solution was made in 50% MeOH in $H_2O$. Working solutions were prepared by diluting the stock solution of the analyte with 50% MeOH in $H_2O$. For standard preparation, three separate weighings were used to prepare primary stock solutions at 26.69, 13.20 and 7.61 μg/ml. A working stock solution at 192.14 ng/ml was prepared from 26.69 μg/ml primary stock solution. Human plasma standard levels 1 to 9, at concentrations of 0.38, 0.77, 1.90, 3.84, 9.13, 15.84, 32.02, 57.71 and 95.02 ng/ml, were prepared by adding the appropriate volume of primary stock solution, or working stock solution into a 50 ml class A volumetric flask and diluting to the mark with normal human plasma with sodium heparin. Standards were then aliquoted into 5 ml polypropylene vials and stored in a freezer maintained at approximately −70 °C. Only one weighing was used to prepared QC primary stock solution at 24.05 μg/ml. Otherwise, quality control solutions were prepared at essentially same manner at concentrations of 0.56, 0.96, 7.97, 31.87 and 79.67 ng/ml.

### 2.3. Sample preparation

Samples were thawed at room temperature, followed by mixing to ensure homogeneity. All steps of sample preparation were handled in automated fashion. Sample transfer steps were accomplished by liquid handler with positive displacement capability (Hamilton Microlab ATplus 2, Reno, Nevada, USA). Each plasma sample (0.300 ml) was loaded into the appropriate well of a clean 96-well polypropylene 2.0 ml plate, followed by the addition of 0.050 ml of working IS solution (approximately 260 ng/ml), 0.040 ml of 600 mM sodium carbonate, and 1.32 ml of extracting solvent (MTBE). The samples were mixed by aspirating and dispensing repetitively on a Tomtec 96-well workstation (Quadra 96 model 320, Hamden, CT, USA). After the plate was centrifuged at approximately 3000 rpm for about 5 min at 10 °C, 1.10 ml of organic layer was transferred from each well to a clean 96-well plate. Then 0.20 ml of 0.1% trifluoroacetic acid was added to the transferred organic layer as the back-extraction solvent and samples were mixed by aspirating and dispensing repetitively again. After phase separation by centrifugation at approximately 3000 rpm the organic layer was removed to a waste plate. The residual organic layer above the aqueous extract was evaporated under a heated stream of nitrogen (approximately 40 °C) for approximately 5 min and an aliquot of the solution was injected into LC/MS.

### 2.4. LC/MS/MS instrumentation

The Shimadzu (Kyoto, Japan) HPLC system included a Shimadzu LC-10 AD HPLC pump, a Shimadzu SCL-$HT_C$ autosampler and system controller. The valves used to control LC flow between mass spectrometer inlet and waste line were from Valco Instruments (Houston, TX, USA). An API-4000 mass spectrometer and computer control system were from PE Sciex (Toronto, ON, Canada). An Aquasil $C_{18}$ 5 μm 3.0 × 150 mm column from Thermo Hypersil-Keystone (Bellefonte, PA, USA) was used as the analytical column. A BHK ODS-P, 5 μm 2.0 × 10 mm cartridge (Naperville, IL) was used as the guard column.

An isocratic HPLC method was employed for separation. Mobile phase consisted approximately 10:90 methanol:0.1% TFA in 10 mM ammonium acetate buffer (v/v). The flow rate for this program was set to 0.6 ml/min. The analytical column was maintained at room temperature of approximately 22 °C and the injection volume was 50 μl.

LC/MS/MS detection was performed using a PE Sciex API-4000 triple quadrupole mass spectrometer with a Turbo Ionspray® ionization source operated in the positive ion mode. The computer control system was Analyst™ version 1.2. The spray voltage was 1500 V. The source temperature was 650 °C. The GS1 (nebulizer gas) setting was 70 and GS 2 (auxiliary gas) setting was 60. Additional parameters of the mass spectrometer acquisition file include DP, 75 V; EP, 12 V; CE, 35 V; CXP, 9 V. The SRM detection channel

for ABT-202 was *m*/*z* 164.0 to 119.0. The SRM detection channel for the internal standard was *m*/*z* 178.0 to 94.0.

### 2.5. Calibration curves and quantitation of samples

Analyst™ version 1.2 was used as the data acquisition and peak area integration. The integration data was imported into Watson® LIMS version 6.2.0.02 for regression and quantitation. 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 weighting of $1/x^2$ (where $x$ is the concentration of a given standard) was used for curve fit. The regression equation for the calibration curve was used to back-calculate the measured concentration at each standard level and the results were compared with the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard level measured.

## 3. Results and discussion

### 3.1. Separation

During method development, a number of silica or polymer based HPLC columns were screened for ABT-202 separation and most of them led to a severely tailing peak and/or a wide peak. This is likely due to the fact that ABT-202 is a primary amine and could easily "stick" to the stationary phase. The addition of TFA to the mobile was found to improve peak shape. Among the columns that gave satisfactory peak shape, an Aquasil $C_{18}$ column was selected for the study mainly because it retains ABT-202 and internal standard better. The presence of TFA in the mobile phase did not deteriorate the signal intensity as much as that observed for certain other types of compounds, such as peptide and proteins, and for ABT 202 only 20% signal reduction was observed when using TFA, instead of other acids such as formic acid or acetic acid.

### 3.2. Extraction

One important consideration in ABT-202 method development was, to minimize potential interference from endogenous plasma compounds, since ABT-202 has a very small molecular weight. The back extraction procedure was developed to deliver a clean extract while maintaining high extraction efficiency by exploiting the analyte's chemical property of being polar and basic. The first extraction with MTBE removes water-soluble components in the plasma, of which most of them are plasma proteins. The second extraction with acidic solution further purifies the analytes from acidic neutral compounds. In order to select the appropriate solvent to extract ABT-202 from plasma, various organic solvents and various combinations of hexane, ethyl acetate, and MTBE were used. MTBE was chosen as the extraction solvent because it gave the highest recovery and the cleanest chromatogram. Formic acid, TFA, acetic acid, phosphoric acid and sulfuric acid were tested as back extraction reagents. TFA and formic acid gave the highest recovery but formic acid was not selected because it forms an adduct with the analyte that creates an additional peak in the chromatogram.

### 3.3. Automation

The Tomtec Quadra™ 96 workstation is equipped with 96 tips and is capable of extracting 96 samples simultaneously. However, the Quadra™ 96 is usually neither accurate nor flexible enough to transfer samples or to add IS. In the current method, a Hamilton Lab AT AT equipped with 12 tips was programmed to aliquot samples from individual tubes to 96-well deep well plates and to add IS. The plate was then brought to the Quadra™ 96 for sample mixing. Typically for LLE using the 96-well plate format, the plates are sealed with a cover and vortex-mixed. Care must be exercised to prevent potential contamination across the wells in this manner. In our method, no heat sealing is necessary. Sample mixing was accomplished by aspirating and dispensing repetitively with the Tomtec 96 well worksation and the potential contamination problem is eliminated. A comparison experiment was conducted to evaluate extraction efficiency between heat-sealing/vortex mixing and Tometec mixing and no major difference was found.

The total extraction time was reduced from an estimated total time of 4 h for the manual operation to 1 h 40 min for the automated operation. The major time saving of using automation was at the sample aliquoting and extraction steps. After back extraction, no sample reconsctitution was necessary, since the sample solvent is compatible with the selected HPLC conditions.

A dual-HPLC with single mass spectrometer configuration was employed to provide a reliable and routine means to increase sample throughput [13]. Dual HPLC systems were set up so that elution of analytes from the first system occurs during the injection dead time of the second system.

A column switching system with a pre-column was used to divert the late eluted interferening components away from the analytical column. The pre-column was regenerated by backwash in every run. With the dual-system approach the analysis time of each run was approximately 4 min.

### 3.4. Selectivity

A specificity test against nicotine was deemed necessary because nicotine has a molecular weight of 162.33 and the $^{13}C$ isotope of its $[M + H]^+$ ion could potentially interfere with ABT-202 analysis. After a solution containing both nicotine and ABT-202 was injected into the LC/MS/MS system using the chromatographic conditions of the assay nicotine was shown to be baseline separated from ABT-202

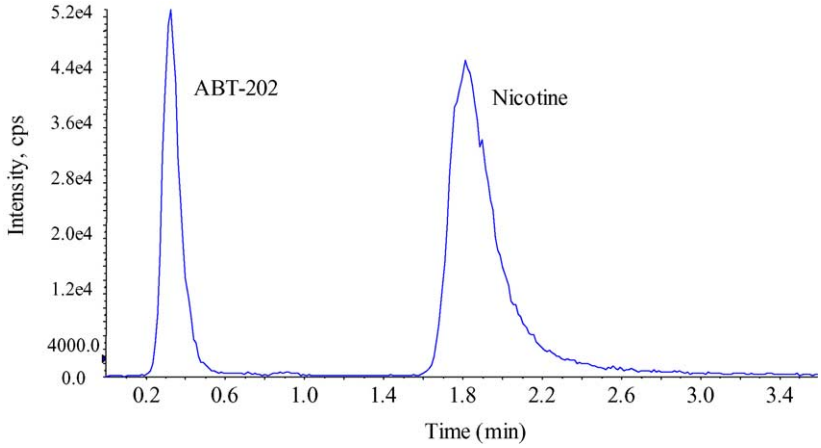


Fig. 2. Reaction ion chromatogram of *m/z* 164.0–*m/z* 119.0 of a mixture of ABT-202 and nicotine in 0.1% TFA. See experimental for chromatographic conditions.

(Fig. 2). To fully evaluate the method specificity against nicotine in plasma analysis, six lots of blank control plasma from smokers, in addition to six lots of blank control plasma from non-smokers, were screened for matrix interferences. None of the 12 lots contained any interference from endogenous components at the retention time of the analyte and IS. Representative chromatograms of an extracted blank sample from a non-smoker and that from a smoker are presented in Fig. 3(a) and (b), respectively.

### 3.5. Calibration curve, precision and accuracy

The precision and accuracy of the method was first validated by three consecutive analytical curves. Each calibration curve contained a single set of calibration standards, six replicates of QCs at each concentration level, six replicates of LLOQ (lower limit of quantitation) evaluation samples, and six replicates of ULOQ (upper limit of quantitation) evaluation samples. Each curve also

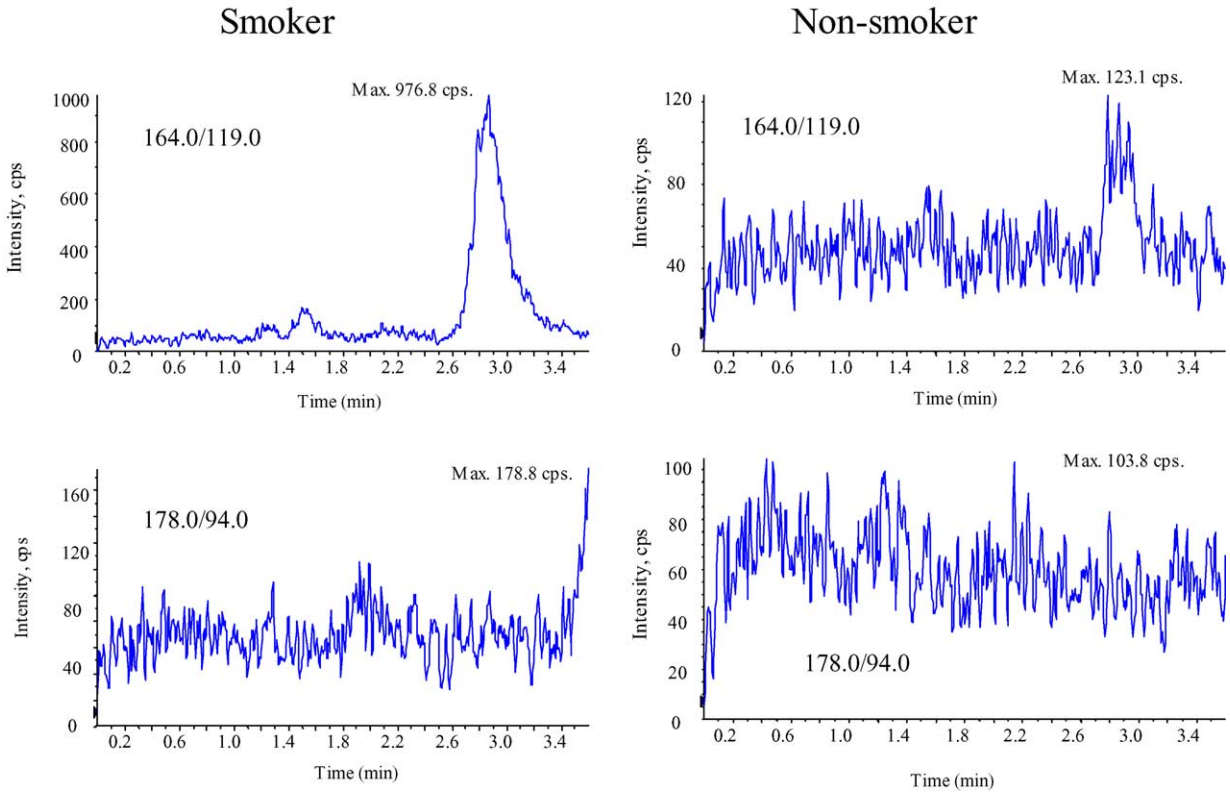


Fig. 3. Representative ion chromatograms of an extracted blank sample from a non-smoker and that from a smoker demonstrating that there was no inteference at the retention times of either ABT-202 or IS. The SRM detection channel for ABT-202 was *m/z* 164.0–119.0. The SRM detection channel for the internal standard was *m/z* 178.0–94.0. Expected retention times for ABT-202 and IS were at 0.7 and 1.8 min., respectively.

contained other test samples such as a system suitability sample.

Statistical data of the calibration curve parameters computed from the three consecutive analytical curves and other validation runs are listed in Table 1. The correlation coefficients of seven calibration curves were all >0.994. The standards show a linear range of 0.38–95.02 ng/ml, using weighted (1/concentration$^2$) least-square linear regression.

The precision and accuracy data for QC samples are summarized in Table 2. The Se data show that this method is consistent and reliable with low % CV and % bias values. For the LLOQ evaluation samples, the % CV ($n = 18$) of the measured concentration was 7.7%. The % bias of the mean of the measured concentrations were –4.7%. The inter-day % CV and % bias of the quality control samples were ≤6.8 and ≤7.6%, respectively.

Representative chromatograms of a LLOQ (0.38 ng/ml) sample and a quality control (7.97 ng/ml) sample are shown in Fig. 4(a) and (b), respectively.

### 3.6. Ion suppression and matrix effect

Matrix effects from co-eluting endogenous components in biological fluids have been well documented in the literature to compromise the reproducibility and accuracy of the analysis [14,15]. To demonstrate that the assay performance is independent from the sample matrix, QC samples were prepared using two different lots of matrix. The QC samples were evaluated using the same calibration curve.

The absolute magnitude of ion suppression by matrix was determined by comparing peak areas of post-extraction spiked samples with neat solutions at corresponding concentrations. For both ABT-202 and IS, the matrix suppression was <10.4% (<10.4% of the analyte signal was suppressed) for low, medium, high concentration samples. The % bias and % CV of the QC samples from two different lots of matrix were from ≤11.8 and ≤11.5%, respectively. The results suggested that matrix effect for the assay was well within the measurement errors.

### 3.7. Recovery

Extraction recovery was determined by comparing the response factors (area/on-column amount) of the appropriate peaks of extracted QC samples with those of post-extraction spiked plasma blanks at similar concentrations. Since, during the extraction only 1.10 ml out of total 1.32 ml of MTBE was transferred, the extraction recovery was therefore corrected for the volume change.

The extraction recoveries of ABT-202 were determined at 0.56, 7.97, 31.87 and 79.67 ng/ml concentrations. The mean recoveries of ABT-202 and IS were 13 and 16%, respectively. Acceptable and consistent recoveries were obtained for both ABT-202 and IS. The low recovery of ABT-202 and IS was not surprising because another similar compound has shown low extraction recoveries during LLE or SPE [1].

Table 1
Summary of calibration curves obtained for the analysis of ABT-202

| | Back-calculated concentration for standards | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | STD 1 (0.38 ng/ml) | STD 2 (0.77 ng/ml) | STD 3 (1.90 ng/ml) | STD 4 (3.84 ng/ml) | STD 5 (9.13 ng/ml) | STD 6 (15.84 ng/ml) | STD 7 (32.02 ng/ml) | STD 8 (57.71 ng/ml) | STD 9 (95.02 ng/ml) | Coefficient of determination ($r^2$) |
| Mean | 0.37 | 0.81 | 1.94 | 3.94 | 9.27 | 15.79 | 32.30 | 55.65 | 89.95 | 0.997 |
| S.D. | 0.01 | 0.04 | 0.06 | 0.15 | 0.31 | 0.29 | 0.72 | 2.58 | 3.63 | 0.002 |
| % CV | 3.5 | 4.8 | 3.3 | 3.9 | 3.4 | 1.8 | 2.2 | 4.6 | 4.0 | 0.2 |
| % Bias | −2.6 | 5.2 | 2. | 2.6 | 1.5 | −0.3 | 0.9 | −3.6 | −5.3 | – |
| *n* | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |

Table 2
Inter-day accuracy and precision of the LLOQ, QC, ULOQ evaluation samples for ABT-202

| | Concentration (ng/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LLOQ (0.38 ng/ml) | Low QC (0.56 ng/ml) | Mid QC (0.96 ng/ml) | Mid QC (7.97 ng/ml) | Mid QC (31.87 ng/ml) | High QC (79.67 ng/ml) | ULOQ (95.02 ng/ml) | Dilution QC (298.51 ng/ml) |
| Mean | 0.36 | 0.54 | 0.96 | 7.57 | 29.45 | 75.35 | 87.87 | 295.92 |
| Inter-run S.D. | 0.03 | 0.04 | 0.06 | 0.52 | 1.67 | 3.81 | 4.47 | 16.04 |
| Inter-run % CV | 7.7 | 6.7 | 6.7 | 6.8 | 5.7 | 5.1 | 5.1 | 5.4 |
| Inter-run % bias | −5.3 | −3.6 | 0.0 | −5.0 | −7.6 | −5.4 | −7.5 | −0.9 |
| *n* | 18 | 42 | 42 | 42 | 42 | 42 | 18 | 15 |

In a typical liquid–liquid extraction procedure, the organic extract is dried down and then reconstituted with HPLC mobile phase. We have compared the extraction recoveries of ABT-202 after single LLE with drying and reconstitution to that from back extraction and the results are similar to each other. During pre-clinical studies of ABT-202, we have developed and validated liquid chromatography–tandem mass spectrometry methods for ABT-202 in mouse and dog plasmas using similar liquid–liquid back extraction procedure with the only major change being the volume of plasma used. All these methods use the same amount (1.32 ml) of extract solvent (MTBE) in a 2.0 ml 96-well plate. We have found that the volume ratio of extract solvent (MTBE) to plasma plays an important role in extraction recovery of ABT-202. For the mouse plasma method, the sample volume was 0.025 ml and the mean extraction recovery was 55.2%. For dog plasma method,the sample volume was 0.050 ml and mean extraction recovery was 41.6%. The result is consistent with the theory that extraction recovery is correlated with the volume ratio of organic vs. aqueous phases. Since, the current method has adequate sensitivity for the concentration determination of ABT-202 in human plasma no further effort was taken to improve the sensitivity.

### 3.8. Integrity of dilution

The effect of dilution without going through a freeze–thaw cycle on the quantitation of ABT-202 was determined. One calibration curve included six replicates of a QC sample (298.51 ng/ml) which was diluted 5-fold with control blank

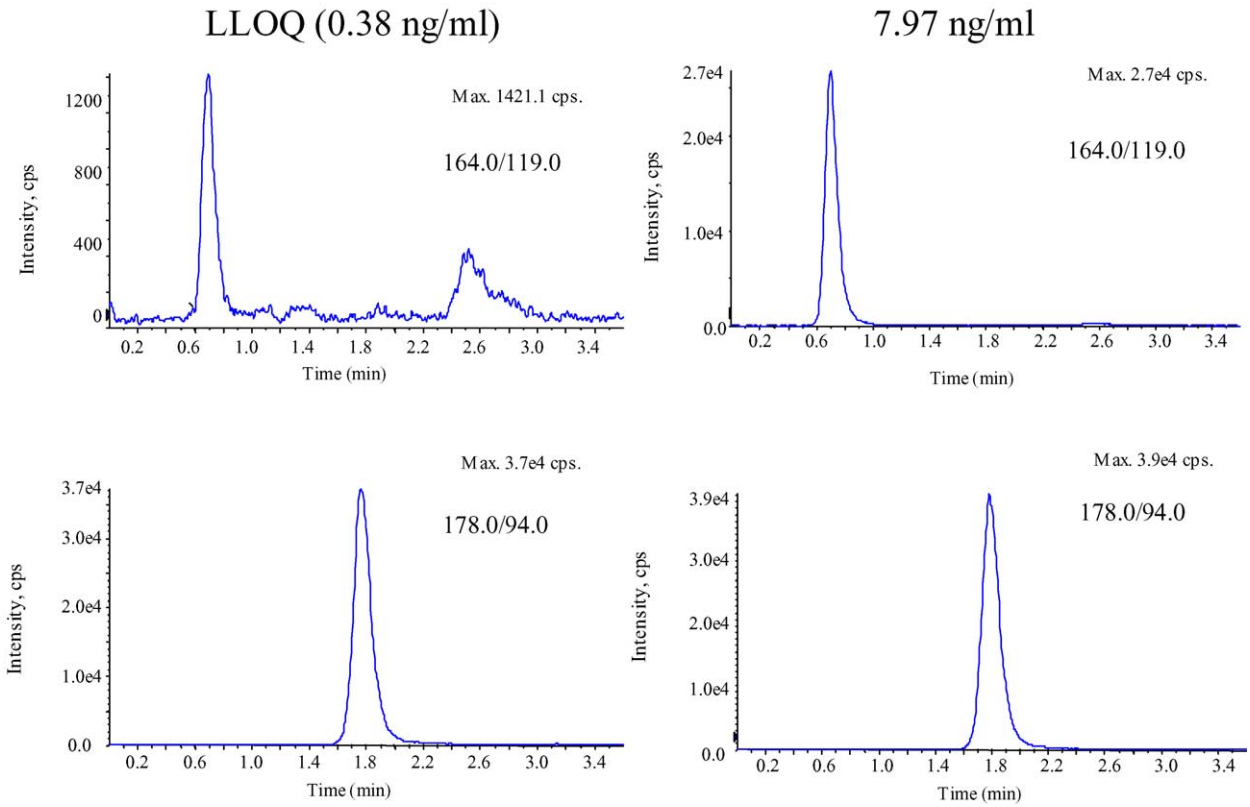


Fig. 4. Representative ion chromatograms of a LLOQ sample and a QC sample at 7.97 ng/ml. The SRM detection channel for ABT-202 was *m/z* 164.0–119.0. The SRM detection channel for the internal standard was *m/z* 178.0–94.0. Expected retention times for ABT-202 and IS were at 0.7 and 1.8 min., respectively.

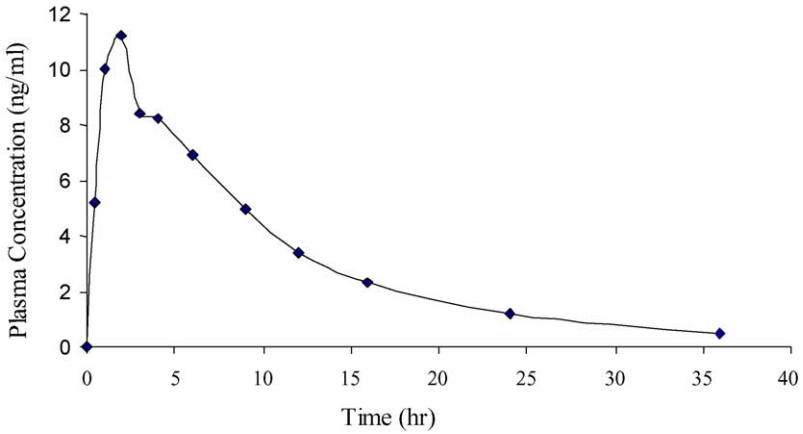


Fig. 5. Plasma concentrations of ABT-202 of a subject following an oral administration of 16 mg dose.

plasma prior to analysis. The dilution procedure was considered to be valid if the % CV and % bias of diluted QC samples were no greater than 15%. For ABT-202, the % CV and % bias for the dilution QC samples were 1.8 and 6.0%, respectively.

### 3.9. Stability

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stability of sample processing (freeze–thaw, bench-top and storage), and chromatography (extracts) were tested and established. The results are summarized in Table 3. Four freeze–thaw cycles and ambient temperature storage of the QC samples for up to 14 h prior to analysis appeared to have little effect on quantitation. QC samples stored in a freezer at −70 °C remained stable for at least 142 days. Extracted calibration standards and QC samples were allowed to stand at approximately 5 °C for 32 h prior to injection and no effect on quantitation of the calibration standards or QC samples was observed. ABT-202 was also determined to be stable in 50% methanolic stock solution stored at 4 °C for 8 days.

### 3.10. Application to clinical studies

The method has been successfully applied to the determination of plasma concentration levels of ABT-202 in support of pharmacokinetic analysis in a Phase I clinical study. A plot of plasma concentration versus sampling time obtained from a subject following an oral administration of 16 mg dose is shown in Fig. 5.

Table 3
Summary of stability of ABT-202

| Matrix | Proven stability | |
|---|---|---|
| | Temperature | Duration |
| Human plasma | ∼−70 °C to ambient | 4 freeze–thaw cycles |
| Human plasma | Ambient | 14 h |
| Human plasma | ∼−70 °C | 142 days |
| Extracts (batch storage) | ∼5 °C | 32 h |

## 4. Conclusion

In conclusion, a sensitive and highly automated 96-well sample extraction method was developed and validated for the determination of ABT-202 in the human plasma. The major advantages of the method are high throughput, efficiency and cost effectiveness. Such method also yields clean extract for sample analysis, which ultimately contributes to the ruggedness of the assay. This LC/MS/MS method for the determination of ABT-202 in human plasma met regulatory requirements for selectivity, sensitivity, goodness of fit, precision, accuracy and stability.

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