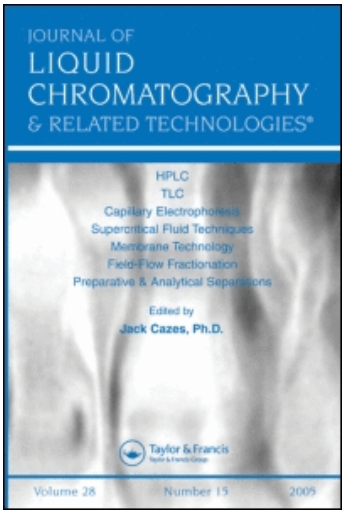

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QUANTITATION OF A NEW MACROLIDE IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRIC DETECTION: COMPARISON WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A method involving high performance liquid chromatography with tandem mass spectrometric detection was developed to determine the concentration of a new macrolide derivative, ABT-229, in human plasma. The analyte was extracted using liquid-liquid extraction with a mixture of ethyl acetate and hexane at basic pH. Detection was highly specific for the analyte of interest, as evidenced by the lack of interference in the chromatograms of drug-free plasma extracts. The lower limit of quantitation was 0.10 ng/mL based on 1 mL of extracted plasma. The linear calibration range of the procedure extended from 0.10 to 199 ng/mL. The method proved reliable and rugged; within-run and day-to-day relative standard deviations were less than 10%. The stability of ABT-229 under frozen storage, room temperature, and freeze/thaw conditions was found to be excellent. Plasma concentrations determined by the method were highly comparable with data obtained by electrochemical detection.

INTRODUCTION

ABT-229 (8,9-anhydro-4"-deoxy-3'-N-ethylerythromycin B 6,9-hemi-acetal) is a macrolide derivative belonging to a new group of compounds called the motilides, which exhibits substantial prokinetic effects but lacks antibacterial activity. During preclinical evaluation, ABT-229 was found to demonstrate significant prokinetic activity in animal pharmacokinetic studies. The observed activity of ABT-229 in animals made it a potential therapeutic agent for further clinical evaluation and was also found to be clinically active in healthy volunteers. During these clinical studies, however, it became evident from the dosages used that sample analysis will require the use of a sensitive and high throughput method.

Initially, a method involving high performance liquid chromatography with electrochemical detection (HPLC-EC) was developed for the quantitation of ABT-229. The method was used successfully for the evaluation of ABT-229 pharmacokinetics in animal models. However, this method proved unsatisfactory for clinical studies because the analysis of a single sample would sometimes require a chromatographic run time of over 80 minutes due to co-extracted interference that required separation.

Pharmacokinetic evaluation of ABT-229 during clinical studies necessitated the development of a higher throughput method because of the large number of samples that are analyzed in each study. Furthermore, because plasma concentrations of ABT-229 were expected to be in the sub-ng/mL range, a sensitive and selective method for the determination of ABT-229 in human plasma samples was also a requirement.

High performance liquid chromatography with mass spectrometric detection has previously been shown to be effective for sensitive and high throughput assays of analytes in biological samples.[1-5] We investigated the use of mass spectrometric detection and subsequently developed a method which involved reverse phase high performance liquid chromatography coupled to atmospheric-pressure chemical ionization with tandem mass spectrometric detection (HPLC-MS/MS). This HPLC-MS/MS method was used for sample analysis in human pharmacokinetic and efficacy studies and is described herein.

As a standard practice in our laboratory, data obtained by two different techniques are usually cross-validated to ensure there are no analytical inconsistencies in measured concentrations of analytes. Therefore, plasma concentrations of ABT-229 measured by the HPLC-MS/MS method are also compared and correlated to data obtained by HPLC with electrochemical detection.

EXPERIMENTAL

Chemicals, Reagents, and Samples

All chemicals and solvents used in the assay were HPLC-grade (Fisher Scientific, Fair Lawn, NJ). Absolute ethanol was USP-grade (Aaper, Shelbyville, KY). Eluent and reconstitution solvents were filtered through a 0.2 μm-pore nylon membrane before use. ABT-229 and internal standard were synthesized and provided by Abbott Laboratories' Drug Discovery group.

A reference solution of ABT-229 was prepared in acetonitrile at a concentration of approximately 1 mg/mL. It was then diluted in an ethanol-water solution, 1:1, v/v, to yield a spiking solution containing approximately 10 μg/mL of ABT-229. Standard and quality control (QC) samples were prepared by independent laboratory staff using reference and spiking solutions from separate weighings. For preparation of standard solutions of ABT-229, pooled human plasma was spiked with the spiking solution for standards. At a concentration of approximately 199 ng/mL, this stock solution of ABT-229 was then serially diluted with pooled human plasma to yield a total of ten standards ranging in concentration from approximately 0.1 to 199 ng/mL. QC samples were prepared similarly by spiking pooled human plasma with the spiking solution for QC samples. With an initial concentration of approximately 159 ng/mL, the stock solution was then serially diluted with pooled plasma, yielding QC samples at four concentration levels: 0.5, 10, 53, and 159 ng/mL which were designated QC Low, QC Midlow, QC Mid, and QC High, respectively.

Extraction

Analytes were extracted from the human plasma matrix by a liquid/liquid extraction technique. After 1.0 mL of human plasma was pipetted into a glass test tube, the internal standard was added: either 50 or 100 μL of ABT-483 (a structural analog of ABT-229; structures of ABT-229 and internal standard are shown in Figure 1) at a concentration of 100 ng/mL in an ethanol-water solution, 1:1, v/v. When the sample had been vortexed briefly, 1.0 mL of an aqueous solution of sodium carbonate (0.5 M) was added, followed by 6 mL of a mixture of ethyl acetate-hexane, 1:1, v/v. The sample was mixed horizontally in a reciprocating shaker at approximately 100 strokes/min for about 5 min. The samples were then centrifuged at about 3000 rpm (relative centrifugal force 1500 *g*) for 10 min at an approximate temperature of 5ºC. The clear upper organic layer was transferred to a silanized glass test tube, with care taken not to disturb the aqueous or interfacial colloid layers with the transfer pipette.

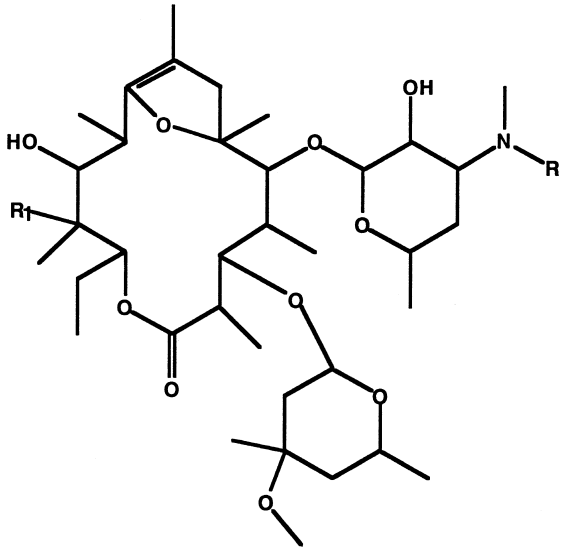

Figure 1. Structures of ABT-229 and internal standard.

The organic extract was then dried in a gentle stream of nitrogen at approximately 37ºC, after which the residue was reconstituted in 75 to 100 µL of acetonitrile-100 mM ammonium acetate solution, 35:65, v/v. Finally, the reconstituted solution was transferred to 300-µL silanized autosampler vials, which were centrifuged briefly at approximately 3000 rpm (relative centrifugal force 1500 × *g*). Samples were stored at 5ºC. The volume of the reconstituted solution injected was 50 µL.

The method was also used for the analysis of dog plasma. The extraction volume was 500 µL for dog plasma, and the volume of the internal standard was 100 µL (100 ng/mL). The mixture was alkalinized with 500 µL of sodium carbonate solution (0.5 M) and extracted with 5 mL of the organic solvent mixture (ethyl acetate-hexane, 1:1, v/v). Residues from evaporated extracts were dissolved in 250 µL of reconstitution solution for HPLC-MS/MS analysis. The volume of the reconstituted solution injected was typically 50 µL. For HPLC-EC analysis, the reconstitution solvent was acetonitrile-0.05 M phosphate buffer at pH 6.5, 35:65, v/v. The typical reconstitution and injection volumes for HPLC-EC analysis were 300 and 100 µL, respectively.

HPLC with Electrochemical Detection

Chromatographic separation of ABT-229 was carried out on a 150 × 4.6 mm YMC Basic column (C_4-C_8 stationary phase, 5 μm, Wilmington, NC, USA). The mobile phase was acetonitrile, methanol, and 0.01 M tetramethylammonium hydroxide in 0.05 M potassium phosphate (pH 6.9), 45:10:45, v/v. An Applied Biosystems (ABI) Spectroflow Model 400 (Foster City, CA, USA) pump was used to deliver the mobile phase at a constant flowrate of 1.0 mL/min. An ESA 5100 electrochemical detector (ESA, Inc., Bedford, MA, USA) was used. Although the retention time of ABT-229 was about 25 minutes, the chromatographic run time was extended by at least an additional 30 minutes to allow the elution of later-eluting peaks that would otherwise interfere with the quantitation of the ABT-229 peak from a subsequent injection. The detector output was digitized by a Beckman ChromLink A/D converter (Beckman Instruments, Allendale, NJ, USA) and processed using a VAX PeakPro system (Beckman Instruments). Calibration curves were derived from the peak area ratio of analyte/internal standard, using least squares linear regression of the area ratio vs. nominal concentration of the standard. Quantitation was performed by calculating the concentration from the peak area ratios of analyte/internal standard using the regression line of the calibration curve.

HPLC with Tandem Mass Spectrometric Detection

Analyte and internal standard peaks were resolved in a reverse-phase column (C_8, 100 × 2.1 mm, 7 μm, Aquapore RP300, Alltech Inc, Deerfield, IL, USA), using a short guard column (30 × 2.1 mm) of similar packing. The mobile phase consisted of acetonitrile-100 mM ammonium acetate solution, 50:50, v/v, with the apparent pH adjusted to 5.5 using glacial acetic acid. The mobile phase was delivered at a flow rate of 500 μL/min using a syringe pump (Model 500D, ISCO, Lincoln, NE, USA). Samples were injected using a CMA 200 Refrigerated Microsampler (Carnegie Medicin, Stockholm, Sweden). Until injection, samples were stored at approximately 5ºC. The typical injection volume was 50 μL, and the chromatographic run time was approximately 3.5 min per injection.

Analysis was performed using a SCIEX API IIIplus triple quadrupole mass spectrometer (Thornhill, Canada), with the HPLC system coupled to the mass spectrometer via the Heated Nebulizer® atmospheric-pressure chemical ionization source. Analytes were ionized in the positive ion mode at a nebulizer source temperature of approximately 450ºC. The orifice voltage was 40 V. Nitrogen (approximately 80 psi) was used as both the nebulizing gas and the auxiliary sheath gas; flow rate was 2 to 4 L/min. The nitrogen for nebulization

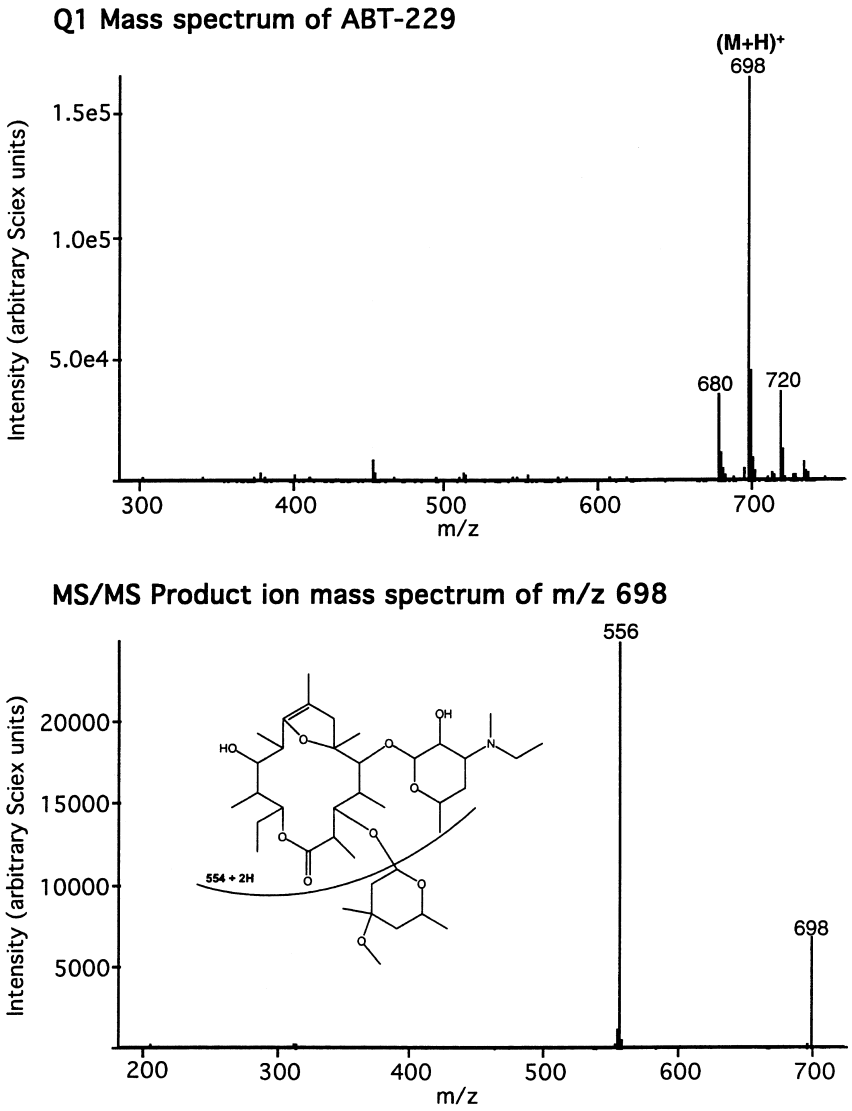

Figure 2. Q1 full-scan mass spectrum of ABT-229 (top) and product ion mass spectrum of m/z 698 (bottom).

and auxiliary flow was produced by a gas generator (NITROX Minigas Generator Model NG 4000, Peak Scientific, Buffalo Grove, IL, USA) connected to an oil-free air compressor (Model 2000, JUN-AIR, Copenhagen, Denmark); the rated purity of the nitrogen was between 95% and 99%, depending on the

flow rate. The curtain gas, delivered at a flow rate of 0.5 to 1.0 L/min, was Ultra High Purity Nitrogen (99.999%, AGA Gas Inc, Maumee, OH, USA). Analyte detection was performed in the selected reaction monitoring mode under collision-activated dissociation conditions.

Fragment ions were produced under collision-activated dissociation (CAD) conditions with the collision gas, Ultra-High-Purity Argon (99.999%, AGA Gas Inc), at a thickness of 150 x 10^{13} molecules/cm^2. A collision energy of 20 eV was used. The precursor-to-product ion reaction of m/z 698 → 556 was monitored for ABT-229; that of m/z 700 → 558 was monitored for the internal standard.

For data acquisition, the dwell time was set at 300 msec, with a pause time of 100 msec. This permitted about 20 to 30 scans across the chromatographic peak, which was generally 30 to 50 sec wide. Peak areas of ABT-229 and the internal standard were calculated using SCIEX MacQuan® software, Version 1.3. Calibration curves were derived from the peak area ratio of analyte/internal standard, using least squares linear regression of the area ratio vs. nominal concentration of the standard. A weighting of 1/*x*, in which *x* is the concentration of a given standard level, was generally found to give an optimal fit to the concentration/response data. Deviations from the regression line were calculated using the regression equation to back-calculate the expected concentration at each standard level. QC sample concentrations were also calculated from these regression curves, using the corresponding analyte/internal standard ratio.

RESULTS AND DISCUSSION

Choice of Analyte-Specific Reactions

A Q1/MS scan of an infusion of ABT-229 and the internal standard (1 μg/mL each) produced essentially only the protonated molecular ions. The molecular ions of the two analytes were subjected to collision-activated dissociation (CAD) with argon atoms; the Q1/MS and MS/MS product ion mass spectrum for ABT-229 is shown in Figure 2. The major fragment ion observed for the analyte and internal standard was the ion resulting from the loss of the cladinose moiety with simultaneous hydrogen transfer (see Figure 2). This loss is consistent with the observation made by Pleasance and co-workers who studied the CAD of erythromycin A and related macrolides.[6] After optimization of source conditions and ion optics, the reaction channels chosen for quantitation were m/z 698 → 556 for ABT-229 and m/z 700 → 558 for the internal standard.

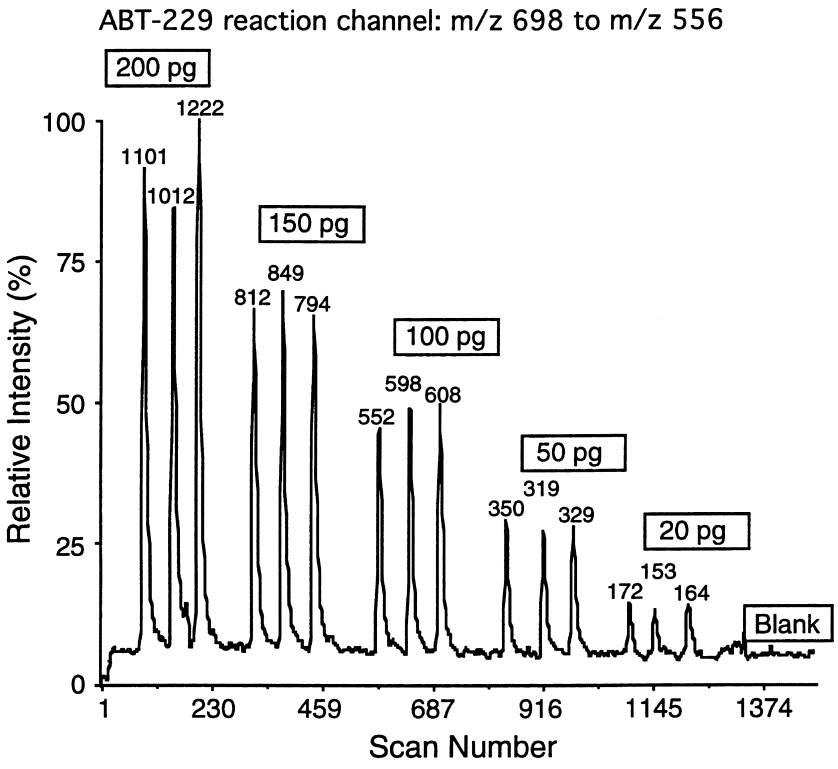

Figure 3. Repeatability of multiple injections (flow-injection analysis) at various concentrations.

The response to triplicate injections of standard solutions of ABT-229 by flow injection analysis was linear, and the relative standard deviation (RSD) of repeat injections was excellent, averaging approximately 6% (see Figure 3). This indicated that the chosen fragmentation channel was sufficiently robust for quantitation.

Analytical Method Performance

The chromatograms from a standard reference solution containing 25 ng/mL of each compound show a small peak in the internal standard channel arising from the (M+2) isotopic contribution from ABT-229 (see Figure 4). Therefore, these compounds needed to be resolved chromatographically to avoid cross-channel contribution between the two compounds.

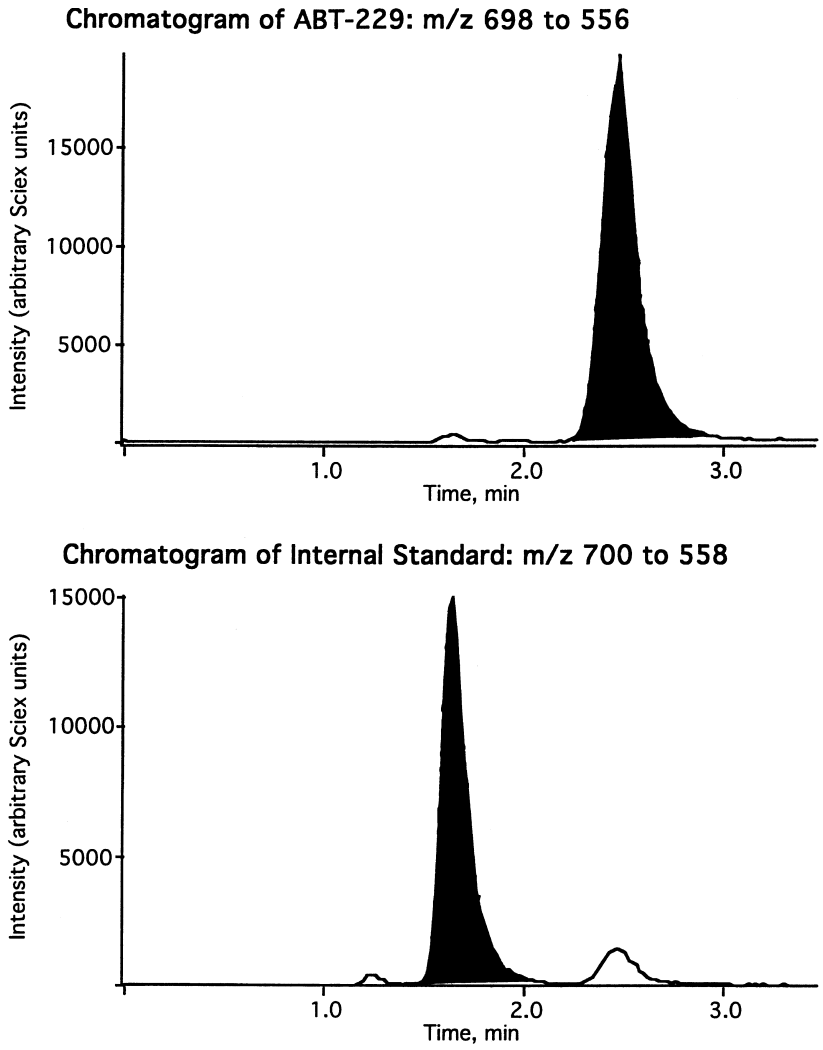

Figure 4. Chromatogram (SRM) of a reference solution containing 25 ng/mL of ABT-229 and internal standard.

In chromatograms from blank human plasma extract, no interfering peaks were observed in either the analyte or internal standard channel which suggested that the selected reaction channels were specific for the corresponding compound.

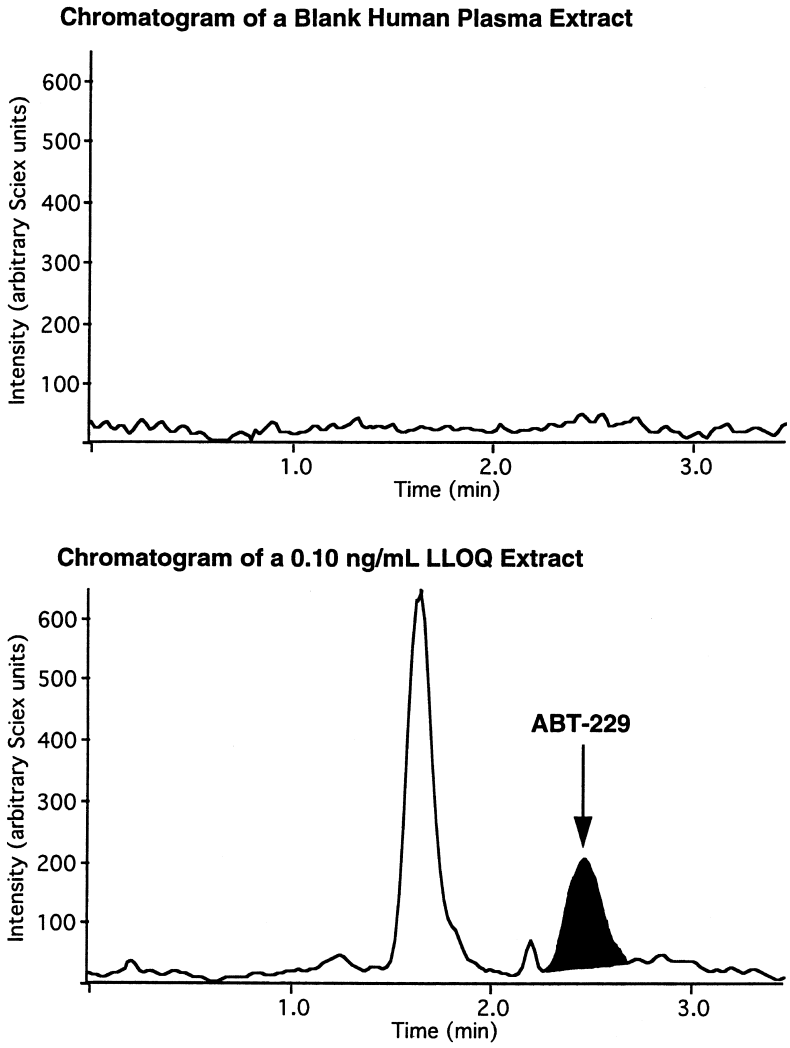

Figure 5. Chromatogram of a blank human plasma extract (top) and an extract of a 0.10 ng/mL human plasma standard (bottom).

Calibration curves consisting of human plasma standards at a range of concentrations were prepared and analyzed to assess the linear concentration range. Standard levels for ABT-229 were determined by assays performed in triplicate on three different days. The lower limit of quantitation (LLOQ) was 0.10 ng/mL (based on 1 mL of extracted human plasma). The accuracy (defined

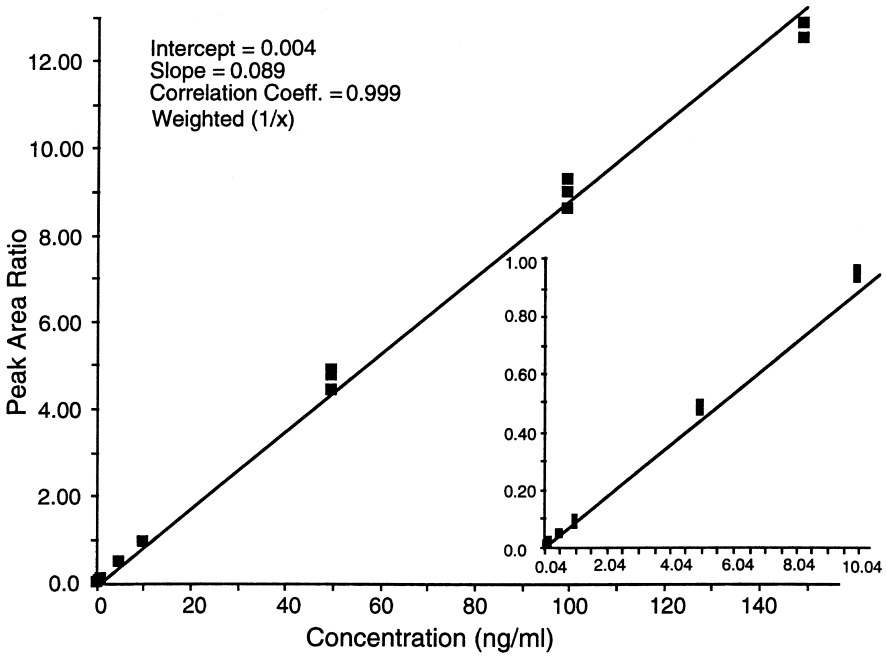

Figure 6. Typical calibration curve showing the linearity in response from 0.1 to 199 ng/mL in human plasma.

as the percent of nominal concentration; calculated from the mean observed concentration/nominal concentration x 100) at the LLOQ was 86% with an RSD of 13% (n=9). Figure 5 shows a typical selected reaction chromatogram of a blank human plasma extract and the chromatogram of the extract observed at the LLOQ of the method. Standard curves of the peak area ratio (analyte/internal standard) vs. analyte concentration were linear over the concentration range of approximately 0.1 to 199 ng/mL in human plasma using a weighting scheme of $1/x$, in which x is the concentration. The correlation coefficients ranged from 0.997 to 0.999, indicating that a linear model is appropriate for response in this concentration range. The day-to-day accuracy for recalculated standard concentrations ranged from 86% to 108% of the nominal concentration and the RSD from 3% to 13%. A typical calibration curve is shown in Figure 6.

Within-run and day-to-day precision and accuracy of the method were evaluated by replicate analysis of QC samples on three different days. The results for ABT-229 QC samples are shown in Table 1. The observed concentrations of individual QC samples ranged from 86% to 108% of the nominal values for QC Low, 93% to 114% for QC Midlow, 91% to 114% for QC Mid, and 90% to 108% for QC High.

Table 1

Within-Run and Day-to-Day Accuracy and Precision of QC Samples

	Nominal Concentration (ng/mL)			
	0.56 (QC Low)	**10.59 (QC Midlow)**	**52.97 (QC Mid)**	**158.90 (QC High)**
Day 1				
Mean	0.50	10.48	53.97	158.86
Accuracy*	91%	99%	102%	100%
RSD	5%	4%	9%	9%
N	4	4	5	4
Day 2				
Mean	0.59	11.31	54.38	150.46
Accuracy*	105%	107%	103%	95%
RSD	3%	5%	4%	3%
N	5	5	5	5
Day 3				
Mean	0.56	11.06	52.35	154.16
Accuracy*	100%	104%	99%	97%
RSD	7%	6%	8%	7%
N	5	5	5	4
Day-to-Day Statistics				
Mean	0.55	10.98	53.57	154.18
Acuracy*	99%	104%	101%	97%
RSD	8%	6%	7%	6%
N	14	14	15	13

* Observed concentrations as percent of nominal concentrations.

None of the concentration deviations exceeded 15% of nominal value among the 56 QC samples analyzed. The respective RSD for each day was low and ranged from 3% to 7% for QC Low, 4% to 6% for QC Midlow, 4% to 9% for QC Mid, and 3% to 9% for QC High. These results suggested that the method had excellent within-run accuracy and precision.

Mean day-to-day concentrations were calculated and found to be 99% of the nominal concentration for QC Low, 104% for QC Midlow, 101% for QC Mid, and 97% for QC High with respective RSDs of 8%, 6%, 7%, and 6%,

indicating the overall reliability and reproducibility of this method in the measurement of ABT-229 concentrations in human plasma. Extraction recovery of the analyte from the plasma matrix was estimated by spiking plasma with a reference solution of ABT-229 and comparing the recovered peak area with that of the reference solution spiked into the organic phase and taken through the evaporation and reconstitution steps.

Spiking, performed at three levels, corresponded to approximately 100, 25, and 5 ng/mL. Recovery was somewhat low: 41% at 100 ng/mL, 54% at 25 ng/mL, and 52% at 5 ng/mL. However, the internal standard appropriately corrected for recovery, so that the method still yielded good precision and accuracy as shown in the analysis of QC samples.

Stability

The stability of ABT-229 in human plasma stored at -20ºC for a period of 6 months was investigated using spiked human plasma samples at three concentration levels corresponding to the low, middle, and high regions of the calibration curve. When the mean concentrations of the stored frozen-stability samples were determined in triplicate, the values ranged from 89% to 97% of the nominal concentrations. Comparison of these data to those available from Day 1 (first day of frozen storage) suggested that ABT-229 was stable when kept frozen at -20ºC.

Plasma samples were also subjected to four cycles of freezing and thawing to determine the in-process stability of ABT-229. The mean concentrations of duplicate assays ranged from 91% to 105% of nominal values of the three concentration levels (low, middle, and high), indicating the stability of ABT-229 under these conditions. The stability of ABT-229 at room temperature was evaluated by exposing the samples to ambient temperature and laboratory lighting conditions for 24 hours after thawing.

Mean concentrations from the three replicate determinations ranged from 97% to 111% of nominal values. The data indicated that the analyte was stable in plasma after exposure to room temperature and laboratory lighting for at least a 24-hour period.

To determine the stability of the analyte in the reconstitution solution, reconstituted solutions from the above experiments was stored at room temperature for approximately 24 to 36 hours, and were then reanalyzed. The mean concentrations of the five replicate determinations ranged from 93% to 99% of nominal values which indicated that the analyte was stable in the reconstituted solution for at least 24 hours.

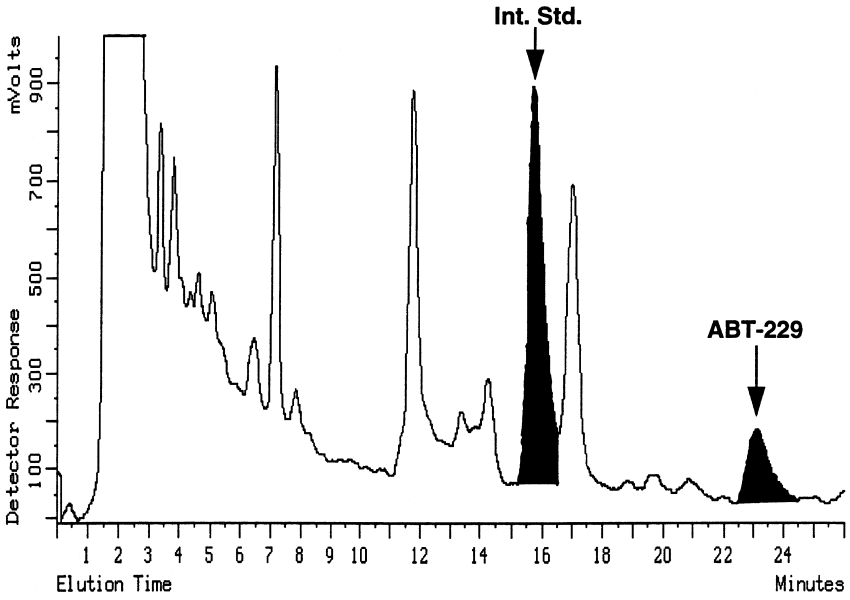

Figure 7. Chromatogram of the extract from a dog plasma standard (23.90 ng/mL) analyzed by HPLC-EC.

Dog Plasma Analysis

The present HPLC-MS/MS method was also used for the analysis of dog plasma samples during toxicokinetic analysis of ABT-229. Chromatograms from pooled dog plasma did not show any interference suggesting that excellent specificity resulting from the selected precursor-to-product ion transition was retained. Analyte extraction recovery for dog plasma was 66% for the spike level 5 ng/mL, 81% for 25 ng/mL, and 88% for 100 ng/mL. Thus, recovery from dog plasma was better than from human plasma, leaving little scope for improvement in extraction efficiency.

In the dog plasma matrix, standard curves were linear in the range of 0.5 to 199 ng/mL. Correlation coefficients ranged from 0.986 to 0.999. There appeared to be no bias toward either end of the curve. Within-run accuracy, based on the mean of triplicate determinations, ranged from 94% to 107% of theory, and RSD ranged from 1% to 17% for the various standard levels. The results of QC determinations demonstrated good within-run accuracy and precision. The accuracy of five replicate determinations was 100% for QC Low (3.97 ng/mL), 99% for QC Mid (39.72 ng/mL), and 98% for QC High (158.90 ng/mL) with an RSD of 7, 6, and 7%, respectively.

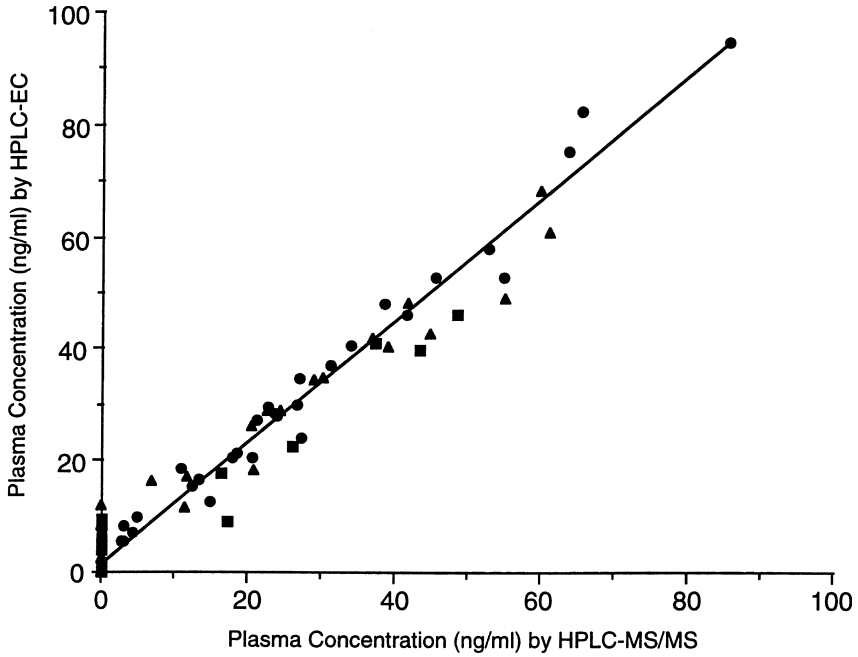

Figure 8. Plot of dog plasma concentrations determined by HPLC-EC and HPLC-MS/MS.

Comparison of Data from HPLC-MS/MS and HPLC-EC

A method involving HPLC-EC was initially used for the quantitation of ABT-229. However, this method proved unsatisfactory for clinical studies because the chromatographic cylce time per injection required at least 80 minutes. The retention time of ABT-229 was about 23 minutes (see Figure 7) but the length of each chromatographic separation had to be extended by an additional 60 minutes due to later eluting electrochemically-active interferences that would otherwise overlap with the ABT-229 peak from the next injected sample. We realized that utilizing a programmed mobile phase gradient elution may shorten the cycle time per injection by forcing the later eluting interferences to elute sooner. However, the selectivity of the method would remain the same and we would not be able to significantly lower the limit of quantitation. Therefore, we chose to improve the selectivity and lower limit of quantitation while simultaneously decreasing sample analysis time by using tandem mass spectrometric detection. As stated earlier, a cross-validation was performed to ensure that there were no discrepancies in measured plasma concentrations by HPLC-EC and by HPLC-MS/MS. Samples obtained from a toxicokinetic study

in dogs, analyzed by the two different methods, yielded very similar measured concentrations (see Figure 8). The correlation coefficient of the fitted line was 0.940 suggesting good correlation. The y-intercept of the line was close to zero with a slope of 0.988, which indicated excellent agreement in the measured concentrations. Small deviations between the two methods were observed at lower concentrations (<10ng/mL), where the HPLC-MS/MS method had better sensitivity and selectivity to distinguish the analyte from potential co-eluting interferences. Significant savings in analysis time was realized by using HPLC-MS/MS as the cycle time per injection was only 3.5 mins for HPLC-MS/MS compared to about 80 mins for HPLC-EC analysis.

CONCLUSIONS

The present method has been used successfully for the analysis of several thousand samples from pharmacokinetic and efficacy studies in human and animals. Although the extraction recovery was not high, the simple liquid-liquid extraction procedure used for sample preparation and preconcentration has proven to be adequate. Overall, the method has demonstrated the level of reliability and ruggedness that are characteristics of a "high-throughput" method.

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