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HPLC–MS/MS determination of a hardly soluble drug in human urine through drug–albumin binding assisted dissolution

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ABSTRACT

ABT-263 is under development for treatment of cancer. In order to support clinical trials, an analytical method for ABT-263 quantification in human urine became necessary. Due to the extremely poor solubility of ABT-263 in aqueous and most common organic solvents, a critical step was to dissolve the drug into urine matrix. Although other potential approaches could be used, addition of powder albumin was found to be the most advantageous. Albumin powder does not significantly alter urine sample volume (≤2.8%) and a range of albumin to urine sample volume ratios can be allowed for full recovery of drug and thus accurate quantification. The procedure is fairly simple and can potentially be a universal approach for compounds with low solubility in urine, but strong protein binding. The method has been validated to support clinical trials.

1. Introduction

Tumor progression, maintenance and even chemoresistance are commonly associated with the over-expression of prosurvival Bcl-2 family members like Bcl-2 and Bcl-xL [1,2]. ABT-263 is an investigational new drug (IND) under development as a potent small molecule inhibitor of Bcl-2 family proteins [3,4]. In preclinical evaluation stage of ABT-263 development, a series of analytical methods for determining drug concentrations in plasma were validated to support animal studies for toxicological evaluations. Although ABT-263 is hardly soluble in water (<0.01 μg/mL) and most organic solvents due to its specific structure, as shown Fig. 1(A), insolubility of ABT-263 turned out not to be an issue in the development of analytical methods in plasma due to strong drug–protein interactions. In clinical development stage, a human urine method becomes necessary for the determination of drug clearance. As a liquid waste product of human body, urine is excreted through kidneys by blood filtration. In addition to water, inorganic salts, urea, creatine, amino acids, peptides, proteins and hormones, enzymes, carbohydrates, etc. are present in human urine. Salts play an important role for the significant reduction of solubility due to the high ionic strength of human urine.

In order to stabilize hydrophobic compounds in urine, a number of approaches have been reported [5–9]. The approaches include addition of a volume of acetonitrile [5], DMSO [6], surfactant [7], and bovine serum albumin (BSA) solution [8], to a certain volume of the urine sample. The volume ratio between urine and additive must be fixed, otherwise the dilution factor of the sample cannot accurately be estimated and consequentially results in inaccurate quantitation. Volume measurement or weighing must be involved to determine how much liquid additive should be added to the samples of unknown volume. In ABT-263 urine method development, most of these reported approaches for hydrophobic drugs in urine were tested and none could fit the need for this compound perfectly. We since have developed a new urine sample treatment method with BSA powder. BSA addition altered urine sample volume less than 2.8%, which was comparable with the natural variation of urine density (up to 3.2%). A broad range of BSA/urine (weight/volume) ratio can stabilize the drug in human urine. Post-treatment of urine samples with powder BSA, the urine samples were analyzed like normal plasma samples with extraction and LC–MS/MS detection [9–11]. The method has been validated according to USA FDA industry guidance for Bioanalytical Method Validation [12] to be applied to support clinical trials.

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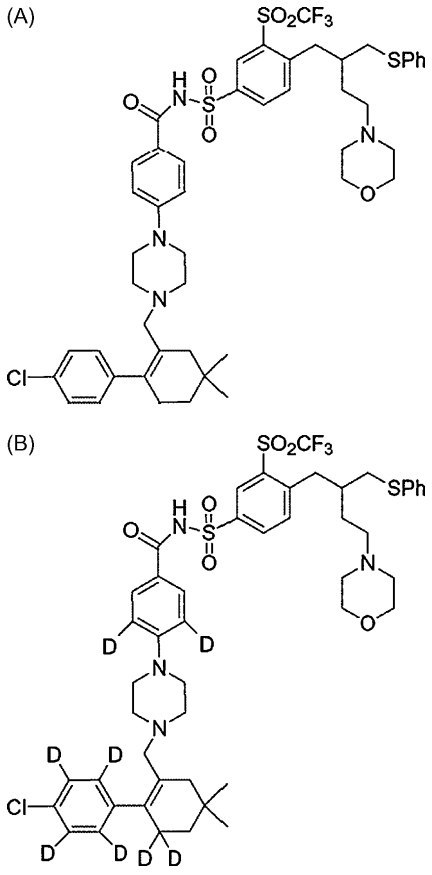

Fig. 1. (A) Structure of ABT-263. (B) Structure of ABT-263 Internal Standard (ABT-263 d_8).

2. Experimental

2.1. Reagents

As a reference standard, ABT-263 was made by Abbott Laboratories (North Chicago, IL, USA). The structure of ABT-263 is shown in Fig. 1(A). Deuterated ABT-263 internal standard (IS) was also prepared by Abbott Laboratories. The structure of ABT-263 IS is shown in Fig. 1(B). HPLC grade acetonitrile and methanol were from EMD Sciences (Gibbstown, NJ, USA). Acetic acid was also from EMD Sciences. A.C.S. grade ammonium acetate was from J.T. Baker (Phillipsburg, NJ, USA). Distilled water was further purified by a Milli-Q water purification system from Millipore (Billerica, MA, USA). Human urine was from Biological Specialties (Colmar, PA, USA). Bovine serum albumin was from Sigma–Aldrich (Milwaukee, WI, USA).

2.2. Instruments

A Microlab AT2 Plus automated liquid handler from Hamilton (Reno, NV, USA) was used during sample preparation. Centrifugation was performed using a Jouan CR412 centrifuge (Jouan, Winchester, VA). HPLC system consisted of a LC-10AD*vp* liquid chromatography pump and a SIL-HTc integrated autosampler and system controller from Shimadzu (Kyoto, Japan). A switching valve between MS and waste from Valco Instrument (Houston, TX, USA) was used to direct solvent flow. An API-4000 mass spectrometer with turbo ionspray (ESI) interface from MDS-Sciex (Concord, ON, Canada) was used as the detector. The mass spectrometer was controlled by Analyst 1.3.2 software from Applied Biosystems (Foster City, CA, USA). The data processing was performed with another computer running Analyst 1.4.2 software from Applied Biosystems as well.

2.3. Chromatography

A Betasil Cyano column (50 mm × 3.0 mm, 5 μm, 100 Å) from Thermo Fisher Scientific (Waltham, MA, USA) was used as the analytical column and a stainless steel frit (0.094″ × 0.062″ × 0.25″ SS) from Upchurch Scientific (Oak Harbor, WA, USA) was used as a pre-column filter. The mobile phase consisted of 1 mM ammonium acetate and 0.1% acetic acid in a mixture of methanol and water in a ratio of 95:5 (v/v). The mobile phase was also used to wash the outside of the autosampler injection needle. Chromatography ran for approximately 2.5 min at a flow rate of 0.5 mL/min. Data collection was started at 1.25 min and lasted for 1.25 min. The HPLC flow was diverted to mass spectrometer only when data was being collected.

2.4. Detection

An API-4000 mass spectrometer with a turbo ionspray source was utilized as a detector for chromatographic elution. The ionization probe was operated at positive ion mode. The SRM channels monitored were m/z 974 → m/z 742 for ABT-263, and m/z 982 → m/z 182 for ABT-263 IS, as shown in Fig. 2. The spray needle voltage was 4500 V, the source temperature was set to 500 °C. Optimized collision energy was 40 eV for ABT-263 and 61 eV for ABT-263 IS. All other parameters of mass spectrometer were optimized with a combined flow infusion of analyte and the mobile phase at 0.5 mL/min.

2.5. Data processing

The data processing was performed using Analyst 1.4.2 software. Only a single set of integration parameters was used to process all data within each run throughout the validation. The processed data was stored in Watson Version 7.2, a Laboratory Information Management System (LIMS) from Thermo Fisher Scientific. The quantitation and statistical analysis were performed in LIMS. A weighting factor of $1/(x{\cdot}x)$ was used in the calibration fitting.

3. Results and discussion

3.1. Method development

The challenges in the human urine method development for ABT-263 were mostly related to the poor solubility of the compound in common solvents except methanol. Due to the poor solubility in water (ng/mL), developing a method to obtain a practical dynamic range for a bioanalytical method in human urine was a difficult task.

In order to prepare homogeneous calibration standards and QCs of ABT-263, one option was addition of solubility enhancing reagents to keep ABT-263 soluble regardless of the differences in matrices and pH. During plasma method development, ABT-263 did not present such a challenge even though plasma was a mostly aqueous substance. Sufficient solubility in human plasma was presumably due to its very high protein binding capability.

Addition of plasma could have been a potential solution. However, plasma would need to be added into the sample collection containers because it would be impossible to obtain a representative aliquot once the sample is transferred into a secondary container. Any transfer prior to the addition of the solubilizing agent could result in a drug loss. In addition, a dilution of drug concentration would result from plasma addition. In order to measure drug concentration accurately, a dilution factor would need to be fixed.

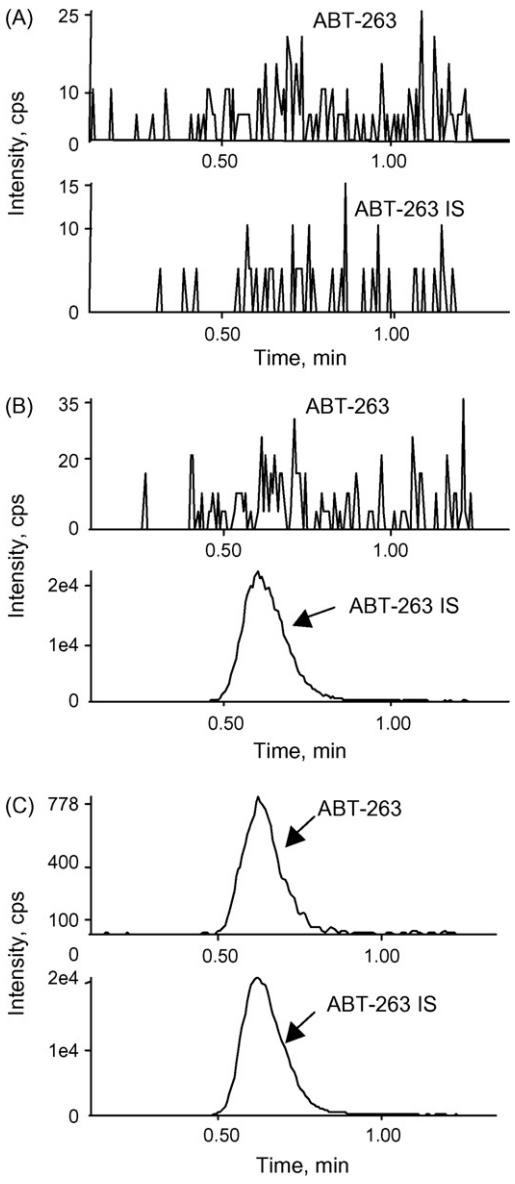

Fig. 2. Representative chromatograms of a blank sample (A), a blank sample with internal standard (B), and an LLOQ sample (0.8 ng ABT-263 on-column) (C). Chromatogram acquisition starts 1.25 min after injection.

In other words, certain volume of plasma would need to be added according to the volume of the urine sample, which implies that multiple volume measurements would be needed. This option also presents some disadvantages such as: the need for a large volume of human plasma to mix with the urine matrix in order to obtain good enough solubility, while plasma to plasma differences might compound the differences from urine to urine and possibly add matrix effects to quantitation. Due to the availability of human plasma, it is also costly to implement this option.

An alternative would be using concentrated albumin solution (such as bovine serum albumin or BSA). This approach would require volume measurements for the urine sample. Also, even with very high concentrations of BSA solutions commercially available (35% BSA), the volume of BSA would need to be at least 10% of the sample volume to obtain the appropriate solubility. Again, multiple volume measurements are needed to ensure accurate results. Powder albumin was found to be the best option for multiple reasons. First, the amount of albumin added to urine in this method contributes less than 2.8% to the sample volume change even up to 66 mg/mL albumin in human urine based on six density determinations from the same lot of matrix. In the albumin contribution experiment, the mean of measured density of blank urine was 1.023 ng/mL and the mean of measured density of urine with albumin added was 1.051 ng/mL. This change of volume is negligible compared with variation of urine sample density determination (up to 3.2% among different lots). Powder albumin could be added into the primary containers for urine collection at collection site or into the confined collection container like that of the anticoagulant added in blood collection. When a range of albumin to urine volume ratio is validated, the amount of albumin addition does not have to be accurately measured based on the volume of the urine samples. Once the method of preparation for standards and QCs using BSA was determined, the next step was the development of the sample extraction procedure. A protein precipitation technique was developed using acetonitrile as the organic solvent to induce protein precipitation. However, the poor solubility of the drug in acetonitrile needed to be addressed, otherwise the drug may not be extracted uniformly at various concentrations. From the physicochemical characterization, it was found that lower pH increases solubility, so in this case precipitation using a 1% addition of acetic acid to the acetonitrile was implemented. In addition to improving drug solubility, the acidified acetonitrile crash helped provide cleaner extract and reduce the amount of impurities being introduced into LC–MS/MS. The Betasil cyano column was chosen due to its selectivity to dipole–dipole interaction and allowance for the appropriate retention of the analyte.

3.2. Method validation

Method has been validated with reference to the Guidance for Industry Bioanalytical Method Validation, USA Food and Drug Administration (May 2001). The validation included evaluation of the linearity of calibration standards, precision and accuracy of quality controls (QC), lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ), matrix effect, selectivity and recovery. The impact of added albumin concentration was evaluated as well. The validation results are summarized as follows.

3.2.1. Sample preparation

Stock solutions used for calibration standard and quality control (QC) preparations were prepared from two independent weighing. Solutions were prepared from solid powders and dissolved with 80:20 (v/v) methanol:water, then they were stored in a refrigerator at approximately 4 °C. In order to spike the drug into human urine with added albumin concentration at approximately 33 mg/mL, the stock solutions were prediluted into working solutions before spiking. Eight calibration standards ranged from 0.0531 to 11.0 μg/mL. Three QC levels ranged from 0.125 to 8.90 μg/mL. For storage, the standard and QC samples were aliquoted into 4 mL polypropylene test tubes, and were frozen at approximately −20 °C until they were used.

3.2.2. Sample extraction procedure

ABT-263 was extracted from the human urine using a semi-automated 96-well protein precipitation technique. The procedure is detailed as follows. Samples were completely thawed at room temperature while protected from light and then mixed well. If there was no albumin added into the urine samples before, the urine sample volume was estimated by visual examination if not already known and albumin was added to ensure albumin/urine volume ratio was within a pre-defined range. 10 μL of internal standard solution were transferred using the repeater pipette to each well of a new 2.2 mL 96-well plate except for the well containing

Table 1
Accuracy and precision of quality controls, LLOQs and ULOQs (nominal concentrations are given in bold)

Run	Calculated ABT-263 concentrations (μg/mL)				
	LLOQ **0.0531**	QC 1 **0.125**	QC 2 **1.12**	QC 3 **8.90**	ULOQ **11.0**
1	0.0569	0.124	1.10	9.40	11.7
	0.0569	0.122	1.09	9.78	10.2
	0.0567	0.122	1.12	9.57	11.5
	0.0548	0.129	1.12	9.02	11.3
	0.0492	0.124	1.08	9.18	10.6
	0.0511	0.114	1.06	9.48	11.0
2	0.0510	0.122	1.06	8.69	11.0
	0.0493	0.118	1.06	9.10	11.2
	0.0547	0.121	1.05	8.66	11.3
	0.0558	0.127	1.11	8.98	11.5
	0.0518	0.124	1.11	9.19	11.1
	0.0535	0.119	1.17	9.22	11.2
3	0.0538	0.118	1.04	9.25	10.9
	0.0532	0.120	1.06	9.05	10.5
	0.0569	0.124	1.12	9.17	11.1
	0.0536	0.122	1.11	9.25	11.5
	0.0613	0.129	1.12	9.61	11.4
	0.0563	0.129	1.17	9.16	11.0
Mean concentration found	0.0543	0.123	1.10	9.21	11.1
Inter-run S.D.	0.00309	0.00413	0.0380	0.290	0.385
Inter-run %CV	5.7	3.4	3.5	3.1	3.5
Inter-run %Bias	2.3	−1.6	−1.8	3.5	0.9
n	18	18	18	18	18

the blank. 10 μL of 80:20 (v/v) methanol:water were transferred using the repeater pipette to the well designated for the blank to compensate the composition. 40 μL of sample were transferred using the Hamilton to each well of the 2.2 mL 96-well plate according to the plate map, then mixed by aspirating and dispensing 6 times 100 μL. 200 μL of 1% acetic acid in acetonitrile solution were added to each well of the 96-well plate, then mixed by aspirating and dispensing 6 times 200 μL. The 96-well plate was centrifuged for approximately 5 min at approximately 3000 × *g* cooled with a setting of 10 °C. 100 μL from each well were then transferred using the Hamilton to a new 2.0 mL 96-well plate. The plate was then dried under a flow of nitrogen heated with a setting of 70 °C. 100 μL of Reconstitution Solution were then added to each well of the 96-well plate. The 96-well plate was capped and vortexed on a multi-tube vortexer for approximately 1 min. 10 μL of the solution in each well were consecutively injected into the LC MS/MS.

3.2.3. Linearity of calibration standards

In validation experiments, total six batches were extracted with 8 calibration standards in each batch. Mean %bias was between −2.8% and 1.4% for calibration standard levels. Minimum calculated coefficient of determination (r^2) was 0.9988 from all six calibration curves.

3.2.4. Accuracy and precision of LLOQs, ULOQs and QCs

In the validation, three consecutive batches were designated for the evaluation of accuracy of QCs, LLOQs and ULOQs. In the evaluation, three concentration levels of QCs to cover the low, mid and high ends of the calibration range, LLOQ as the lowest calibration standard and ULOQ as highest calibration standard were extracted in six replicates with the calibration standards in each batch. A total of 54 QCs, 18 LLOQs and 18 ULOQs were extracted and all of them were within the acceptance criteria. The results are shown in Table 1. The nominal concentrations were shown in bold in Table 1. Overall %CV was not greater than 3.5% and mean %bias was between −1.8% and 3.5% for all QCs. %CV was 5.7% and mean %bias was 2.3% for LLOQs. %CV was 3.5% and mean %bias was 0.9% for ULOQs.

3.2.5. Matrix effect

In order to evaluate the matrix effect, six matrix effect quality controls were prepared at a concentration close to the low QC by spiking drug into six individual human urine lots with added albumin at a concentration of approximately 33 mg/mL. The pH of the individual lots of urine ranged from 5 to 8. Six replicates for each lot were extracted, then the concentration was calculated against the calibration curve. The calculated concentrations were compared with the nominal values from spiking for the calculation of %bias. In the six lots tested, all 36 replicates were within 15% of the nominal concentration, and the mean % bias for the individual lots was between −7.0% and 9.4%.

3.2.6. Selectivity

In order to demonstrate the selectivity of this method, six lots of blank matrix with and without IS were screened for interference from endogenous matrix components. No interference was observed. All blank samples had undetectable peaks at the retention time of the LLOQ. A typical chromatogram of a selectivity sample without IS is shown in Fig. 2(A). The deuterated internal standard did not contribute to the ABT-263 peak area and that can be seen in Fig. 2(B), which shows the chromatogram of a blank sample with internal standard. Fig. 2(C) is the chromatogram of an LLOQ sample for comparison. The LLOQ signal is sufficiently above the noise level to provide a distinctive limit of quantitation. In addition, the carryover was tested using a blank sample with internal standard injected immediately after a high standard. The carryover was found to be less than 0.1% by peak height.

3.2.7. Extraction recovery

In order to evaluate the extraction recovery, recovery controls (RC) were prepared in 80:20 (v/v) methanol:water at three different concentrations. Recovery evaluation (RE) QCs were also prepared at three similar concentrations in matrix with albumin. In the evaluation experiment, 40 μL of a RE QC sample were treated as a normal sample (i.e. internal standard and extraction solvent were added) and were extracted into the injection plate as in the sample extraction procedure. In some other designated wells of the 96-well plate, 40 μL of matrix (blank urine with albumin) were treated as a normal sample (i.e. internal standard and extraction solvent was added) and were extracted into the injection plate as in the sample extraction procedure. Prior to drying 40 μL of RC solution were added to the wells of the injection plate, then dried and reconstituted as normal. Extraction recovery was calculated by comparing RE QC and RC samples at three concentration levels. Measured extraction recovery was between 37.5% and 42.1% (based on approximately 40% transfer of extracts). The extraction recovery of ABT-263 IS was also measured in the similar way, measured recovery was 46.4%.

3.2.8. Impact evaluation for added albumin concentration

In order to evaluate the impact of added albumin concentration, various amounts of albumin were added into the quality control samples prepared by spiking drug into individual polypropylene tubes that were then capped. By considering the volume variation of sample collection, different albumin/sample volume ratio was evaluated at two different concentrations similar to the low and high quality control samples. The urine sample volume range was from 1.0 to 4.0 mL. The final concentration of albumin was between 33 and 66 mg/mL. The variation in concentration of albumin added to unknown samples of various volumes was found to be sufficient to cover the possible ratio among urine volume, albumin and drug concentration. Acceptable values show the results are not affected

significantly regardless of the amount of albumin added if within the validated range of 33–66 mg/mL. Mean bias for three replicates tested at each level was between −8.7% and 0.4% for the samples with a final albumin concentration of approximately 66 mg/mL and −14.2% and 0.1% for the samples with a final albumin concentration of approximately 33 mg/mL albumin. All tested samples were measured within 100 ± 15% of their nominal concentrations.

3.2.9. Stability of urine samples

The freeze–thaw and short-term room temperature were tested using evaluation QC samples with albumin added during the QC preparation prior to initial freezing and also ones with albumin added after freezing and prior to analysis. The first scenario could cover the samples collected in the containers with albumin added at the collection time and then stored, and the second scenario could cover the samples collected in the containers without albumin added and stored. For the evaluation of QC samples with albumin, sets of quality control samples were treated with at least three simulated freeze/thaw cycles to cover assay, possible re-assays or even technical thawing like freezer failure, etc. QC samples without albumin added were evaluated for only two additional freeze–thaw cycles to cover the necessary freeze/thaw and possible technical thawing. The first cycle evaluation QC samples were frozen for a minimum of 24 h at −20 °C. All subsequent evaluation cycles were frozen at −20 °C for at least 12 h between thaws. Samples were thawed completely under the intended condition, held at room temperature for a documented period of time and returned to the freezer at approximately −20 °C for storage. A record was kept to determine the approximate time the samples were at room temperature. The concentration of the evaluation samples was computed using the calibration curve. The concentration of the evaluation samples was compared to the concentration of the control samples, which were samples that experienced their first thaw on the day of analysis. Mean bias was calculated by comparing the determined concentration of evaluation QC samples and the determined concentration of control samples. A mean bias within ±15% was used as an acceptance criterion. Freeze/thaw evaluation QC samples with albumin added were stable for at least 4 freeze/thaw cycles in polypropylene cryogenic vials protected from light and were exposed to room temperature for at least 77 h. Freeze/thaw evaluation QC samples without albumin were stable for 3 freeze/thaw cycles in polypropylene cryogenic vials protected from light and were exposed to room temperature for at least 24 h.

Frozen storage stability was also evaluated using both quality control samples with and without albumin added at preparation. Stability was established for a period between the initial evaluation and a later evaluation of samples from the same preparation using a fresh calibration curve. All calculated concentrations were compared to the nominal concentration. Frozen storage stability was established for at least 41 days of storage in polypropylene cryogenic vials at −20 °C for evaluation samples with albumin, and at least 34 days of storage under the same conditions for samples without albumin.

4. Conclusion

Albumin powder was used as a new solubility-enhancing additive for human urine sample preparation. This novel approach is simple. Unlike organic solvents, human plasma or albumin solution, albumin powder does not significantly alter the urine sample volume. Albumin can be added similar to the way anticoagulant is added in blood collection improving the representative sampling of urine from a block container into sample tubes. Because a range of albumin to urine sample volume ratio is allowed, the approach is simple and the quantity of albumin does not need to be precise for each sample. The treatment using this approach has been successfully used to stabilize a poorly soluble drug (ABT-263) in human urine. The method has been validated to support clinical trials. Proper addition of albumin into the sample collection containers within validated albumin/urine volume ratio range greatly improves drug recovery and enables reproducible sampling for accurate quantitation of the drug. This approach represents a new way to stabilize strong protein-binding drugs with low aqueous solubility in urine samples.

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