Quantitation of Motexafin Lutetium in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry and Inductively Coupled Plasma-Atomic Emission Spectroscopy

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ABSTRACT

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) methods were developed and validated for the evaluation of motexafin lutetium (MLu, lutetium texaphyrin, PCI-0123) pharmacokinetics in human plasma. The LC-MS/MS method was specific for MLu, whereas the ICP-AES method measured total elemental lutetium. Both methods were fast, simple, precise, and accurate. For the LC-MS/MS method, a closely related analogue (PCI-0353) was used as the internal standard (IS). MLu and the IS were extracted from plasma by protein precipitation and injected onto an LC-MS/MS system configured with a C18 column and an electrospray interface. The lower limit of quantitation was 0.05 $\mu\text{g MLu mL}^{-1}$, with a signal-to-noise ratio of 15:1. The response was linear from 0.05 to 5.0 $\mu\text{g MLu mL}^{-1}$. For the ICP-AES method, indium was used as the IS. The sample was digested with nitric acid, diluted, filtered, and then injected onto the ICP-AES system. Two standard curve ranges were validated to meet the expected range of sample concentrations: 0.5 to 50, and 0.1 to 10 $\mu\text{g Lu mL}^{-1}$. The LC-MS/MS and ICP-AES methods were validated to establish accuracy, precision, analyte stability, and assay robustness. Interday precision and accuracy of quality control samples were $\leq 6.3\%$ coefficient of variation (CV) and within 2.2% relative error (RE) for the LC-MS/MS method, and $\leq 8.7\%$ CV and within 4.9% RE for the ICP-AES method. Plasma samples from a subset of patients in a clinical study

were analyzed using both methods. For a representative patient, over 90% of the elemental lutetium in plasma could be ascribed to intact MLu at early time points. This percentage decreased to 59% at 48 hours after dosing, suggesting that some degradation and/or metabolism of the drug may have occurred.

KEYWORDS: motexafin lutetium, LC-MS/MS, ICP-AES, pharmacokinetics, lutetium texaphyrin

INTRODUCTION

Texaphyrins are aromatic, pentadentate, porphyrin-like macrocyclic ligands that contain an expanded central core capable of forming highly stable 1:1 complexes with large cations such as trivalent lanthanides.¹⁻⁵ Motexafin lutetium (MLu) is the texaphyrin formed when Lu (III) is inserted into the texaphyrin macrocycle (**Figure 1**).⁶ When activated using far-red light (720-760 nm), MLu, a photosensitizer, produces highly toxic singlet oxygen capable of destroying diseased tissue.⁷⁻¹⁰ MLu is currently undergoing clinical evaluation for the photodynamic treatment of tumors, atherosclerosis, and age-related macular degeneration.^{2,11-17}

Both clinical and nonclinical studies have been undertaken in order to elucidate the effect of dose on the pharmacokinetics and/or efficacy of MLu in biological systems. Bioanalytical methods developed in support of these studies have used scintillation counting (for studies using ¹⁴C-radiolabeled compound⁶), total fluorescence,¹⁸⁻²¹ and high-performance liquid chromatography (HPLC).^{6,22}

This report describes the development and validation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) methods for the

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Figure 1. Structures of MLu (A) and internal standard (B).

evaluation of MLu pharmacokinetics in human plasma. The LC-MS/MS method was developed specifically for MLu and is particularly advantageous compared to other HPLC methods because the mass spectrometer provides an extra degree of selectivity for MLu. This

added selectivity reduces the possibility that any degradation products or metabolites of MLu will interfere with MLu quantitation. If new metabolites of MLu are subsequently discovered in plasma, an LC-MS/MS method is generally easier to adapt for simultaneous

quantitation of these new metabolites, since chromatographic separation of the metabolites may not be required. In addition, the LC-MS/MS method has a run time of 3 minutes, which reduces the analytical instrument time nearly 7-fold when compared to HPLC using a standard absorbance detector.²²

The ICP-AES method measures the total amount of elemental lutetium in plasma. Because the plasma sample is digested before analysis, lutetium derived from intact MLu, metal-containing metabolites/degradation products, lutetium complexes with other molecules in plasma, and unbound lutetium are all included in the total amount of lutetium measured. If a good correlation between ICP-AES and LC-MS/MS methods can be established, ICP-AES may provide a rugged alternative to LC-MS/MS for estimating the amount of MLu in samples destabilized by exposure to light and/or inadequate storage conditions. ICP-AES-based methods would also be useful in a mass balance study in which the total recovery of Lu (III) in urine and feces is measured as a percentage of the administered dose.

Individual assays for MLu and total elemental lutetium in human plasma provide important information that is needed for a more complete understanding of MLu pharmacokinetics. If the total amount of elemental lutetium in plasma can be accounted for as intact MLu, it would demonstrate stability of the compound in the body. If more elemental lutetium is found in plasma than can be attributed to MLu, it would suggest that the drug has been metabolized and/or degraded and could provide important information on the molecular species of Lu (III) in circulation after dosing. Since the biological activity of texaphyrins is dependent on the central metal cation, any process that causes Lu (III) to be dissociated from MLu could alter the activity and/or biodistribution of the compound. Another group has applied a similar approach for quantitation of gadodiamide and gadolinium in serum by HPLC and ICP, respectively. Their results showed good agreement between total gadolinium and gadodiamide levels in clinical study samples, indicating the absence of significant metabolites in serum for gadodiamide.²³

Both the LC-MS/MS and the ICP-AES methods are ideal for use in preclinical and clinical studies. These assays require small amounts of plasma, use a simple sample preparation procedure, and have short run times. A full validation of both methods resulted in assays that were sensitive, accurate, rugged, and reproducible. Trending analysis revealed that accuracy and reproducibility were maintained throughout months of clinical studies. The utility of the LC-MS/MS and ICP-AES methods was demonstrated by using both assays

to analyze each plasma sample from a single representative patient.

MATERIALS AND METHODS

Instrumentation

LC-MS/MS

An Applied Biosystem MDS Sciex (Concord, Ontario, Canada) API 365 LC-MS/MS system was used. The analytical column was a Zorbax Eclipse XDB-C18 column, 150 × 3.0 mm (3.5 μ m), from Agilent (Palo Alto, CA). The precolumn was BDS Hypersil C18, 10 × 2 mm (3 μ m), from Keystone (Bellefonte, PA), which was changed before each batch run. The mobile phase was acetonitrile-ammonium acetate (100 mM, pH 4.3) 40:60 (vol/vol). The flow rate was 0.5 mL min⁻¹. The autosampler was a Waters 717 (Milford, MA). The needle wash was acetonitrile-water 50:50 (vol/vol). The run time was 2.5 minutes. Turbo Ionspray (Concord, Ontario, Canada) was used for the interface. The gas flow of the Turbo IonSpray was 8 L min⁻¹ with gas pressure at 80 psi. Positive ions were monitored in the multiple reaction mode (MRM) with mass-to-charge ratio (*m/z*) of 1106.4 → 1106.2 for MLu, and of 1150.4 → 1150.2 for the internal standard (IS). The dwell time was 200 msec for both MLu and the IS. The injection volume was 5 μ L.

ICP-AES

A PerkinElmer (Norwalk, CT) Optima ICP-AES instrument model 3000 XL was used. The radio frequency (RF) power was set at 1300 W, nebulizer flow at 0.500 L min⁻¹, auxiliary flow at 0.7 L min⁻¹, and plasma flow at 15 L min⁻¹. High-purity argon gas was used with head pressure at 80 psi. A cross-flow pneumatic GemTip nebulizer, model N058-0613, was used. The peristaltic pump from Cole Palmer (Vernon Hills, IL), Masterflex Model 7520-25, was set at a 600-speed setting. The autosampler was a Cetac ASX-500 from Cetac Technologies (Omaha, NE). A rinse solution of 10% nitric acid was used. Temperature was maintained at 16 C with a Coolflow CFT-33 refrigerated recirculator from Neslab (Portsmouth, NH). The element Lu261 at peak wavelength of 261.54 nm was read with a peak window of 0.0241; the upper interval was set to 0.040, and the lower interval was set to 0.048. The peak wavelength of the IS element indium was 325.609 nm and read with a peak window of 0.0297; the upper interval was set to 0.050, and the lower interval was set to 0.040. The viewing height was set at 15 nm, sam-

Figure 2. Synthetic scheme for the synthesis of the PCI-0353 IS.

pling time at 2000 msec, and integration time at 35 msec.

Materials

MLu

MLu (PCI-0123), lot SA701, purity 91.3%, was supplied by Pharmacyclics, Inc (Sunnyvale, CA).

PCI-0353, Lot 202-050

The PCI-0353 IS (bis(acetato-*O*)[9,10-diethyl-20-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-4,15-dimethyl-21-(3,6,9,12-tetraoxatridec-1-yloxy)-8,11-imino-3,6:16,13-dinitrilo-1,18-benzodiazacycloeicosine-5,14-dipropanolato- $N^1, N^{18}, N^{23}, N^{24}, N^{25}$] lutetium hydrate) was prepared similarly to published methods for texaphyrin synthesis^{6,24} (**Figure 2**). Briefly, the non-aromatic macrocyclic ligand (3.0 g, 3.1 mmol), Lu (III) acetate hydrate (1.67 g, 3.91 mmol), and triethylamine (3.5 mL, 25 mmol) were mixed together in methanol (600 mL) and heated to reflux while left exposed to air.^{6,24} The general procedure previously reported was used,^{6,24} and the reaction was deemed complete after 7 hours; workup and recrystallization from ethanol/isopropyl alcohol (IPA) afforded 1.9 g (51%) of PCI-0353 as a microcrystalline green solid. For PCI-0353: UV/visible: [(MeOH) $\lambda_{\max, nm}$ (log ϵ) for Soret-type and Q-type bands, respectively]: 476 (5.09), 732 (4.60); electrospray mass spectrometry, $[M - OAc]^+$: m/z 1150.4; analysis calculated for $[C_{50}H_{70}LuN_5O_{11}](OAc)_2 \cdot H_2O$: C, 52.81; H, 6.40; N, 5.70; Lu, 14.25. Found: C,

52.74; H, 6.26; N, 5.55; Lu, 14.37. The purity of PCI-0353 was checked using HPLC analysis (ie, >90% relative purity) using a similar method previously reported for MLu.⁶

UV-visible spectra were obtained on a Hitachi-U3000 spectrophotometer (San Jose, CA). The relative purity was determined by HPLC analysis on the Waters Alliance HPLC system with Millennium Software (Milford, MA). Electrospray ionization mass spectrometry analysis was carried out on a Agilent HPLC 1100 (Palo Alto, CA) with a Thermo-Finnigan LCQ ion trap mass spectrometer (San Jose, CA). Schwarzkopf Microanalytical Laboratory (Woodside, NY) performed the elemental analysis of PCI-0353. All solvents were of reagent-grade quality and purchased commercially. Lutetium acetate was purchased from Alfa AESAR (Ward Hill, MA).

Other Materials

Lu and Indium (In) ICP standards for the ICP-AES analysis were purchased from GFS Chemicals (Powell, OH). All chemicals used were analytical or HPLC grade. The organic solvents, concentrated nitric acid, and 30% hydrogen peroxide were from Fisher (Fair Lawn, NJ). Glacial acetic acid, ammonium acetate, and HPLC-grade water were from Mallinckrodt (Paris, KY). Mobile phases were vacuum-filtered through 0.45- μm nylon membranes from Gelman Sciences (Ann Arbor, MI). Deionized water was prepared in-house using a NANOpure water purification system from Barnstead (Dubuque, IA). Ethylenediamine-

tetraacetic acid, disodium salt (EDTA- Na_2) and heparin-treated plasma, serum, and heparin-treated whole blood for control samples were purchased from Nashville Biological (Cincinnati, OH) and Biochemed Pharmacologicals, Inc (Winchester, VA).

Preparation of Solutions

LC-MS/MS

All solutions of MLu were prepared in polypropylene containers to avoid adsorption of MLu to glass. Primary stock solutions for standards and quality control (QC) samples were prepared from separate weighings. Each primary stock was a 1 mg mL^{-1} solution of MLu in 40/60 vol/vol methanol/25mM pH 5.2 ammonium acetate buffer. The high standard (5.0 $\mu\text{g mL}^{-1}$) was prepared in human control EDTA plasma by 200-fold dilution of a 1 mg mL^{-1} primary stock. Subsequent standards of 4.0, 2.0, 1.0, 0.50, 0.20, 0.10, and 0.05 $\mu\text{g mL}^{-1}$ were prepared by further dilution of the 5.0 $\mu\text{g mL}^{-1}$ standard with control plasma. The high QC sample (3.75 $\mu\text{g mL}^{-1}$) was prepared by dilution of a primary stock solution with human control plasma. The middle- and low-QC samples at 0.75 and 0.15 $\mu\text{g mL}^{-1}$ were prepared by further dilution of the high-QC sample with human plasma. Aliquots of standards and QC samples were distributed into polypropylene tubes and stored at -70°C .

ICP-AES

The method was developed and validated for samples in human EDTA plasma and further validated to include heparinized plasma, serum, and heparinized whole blood. A series of working standards was prepared by dilution of a 1000-ppm Lu ICP standard with water. These solutions were stored at 2 to 8°C in polypropylene tubes and were diluted 10-fold before analysis with control plasma, serum, or whole blood. Two standard ranges were used. The high-range standard concentrations were 50, 40, 30, 20, 10, 3.0, 1.0, and 0.5 $\mu\text{g mL}^{-1}$; and the low-range standard concentrations were 10, 8.0, 6.0, 4.0, 2.0, 0.6, 0.2, and 0.1 $\mu\text{g mL}^{-1}$. QC samples were prepared in volumetric flasks from multiple dilutions of a 10 000 ppm Lu ICP standard with control matrix at levels of 37.5, 25.0, 15.0, 7.5, 3.0, 1.5, and 0.3 $\mu\text{g mL}^{-1}$. They were aliquoted into polypropylene tubes and stored at -20°C . For the IS, a 10 000-ppm indium ICP standard was used without dilution for the high standard range and diluted to 2000 ppm with water for the low standard range.

All QC samples were stored with the clinical samples until assayed. Some QC samples were reserved and stored separately for long-term storage stability tests. A set of calibration standards were placed near the beginning of each run before at least six replicates of QC samples, which were scattered among test samples (eg, tests of recovery, stability, selectivity). The length of some of the validation runs was designed to mimic the length of a clinical sample analysis.

Sample Processing Procedures

LC-MS/MS

Samples were processed under yellow light. Exactly 200 μL of each QC sample, analytical sample, or standard was added to a 1.5-mL microcentrifuge tube. After 20 μL of the IS and 0.8 mL of 1M acetic acid in acetonitrile were added, the tubes were vortexed for approximately 15 seconds, then set in a refrigerator for 30 minutes. They were then centrifuged for 15 minutes and transferred onto the LC-MS/MS in amber autosampler vials with plastic inserts for injection.

ICP-AES

Each QC sample, analytical sample, or standard was pipetted (0.5 mL) into a 16 $\times$ 100 mm screw-cap tube. After the addition of 50 μL of the IS, the solution was mixed and 1 mL of concentrated nitric acid was added, followed by 0.5 mL of 30% hydrogen peroxide. The tubes were digested for 60 minutes at 120°C . After cooling, 6 mL of water was added and the contents were vortexed and filtered through SA720 filter paper (Schleicher & Schuell, Keene, NH) into a 13 $\times$ 100 mm tube, in preparation for injection onto the ICP-AES.

Data Regression

The LC-MS/MS chromatograms were integrated using MacQuan software from Applied Biosystem MDS Sciex. For ICP, integration of the emission counts was performed on the PerkinElmer ICP-AES with a Digital DEC PC. All integrated data were transferred into a VAX/VMS Oracle database for regression. The peak-area ratio of analyte to the IS (y) was plotted against analyte concentration (x). A linear regression with 1/x weighting was used to determine slopes, intercepts, and correlation coefficients. Unknowns were calculated by the following equation: $x = [y - (y\text{-intercept})]/\text{slope}$. For ICP, the weighting was $1/x^2$. The conversion of concentration units from $\mu\text{g mL}^{-1}$ Lu to $\mu\text{g-equiv mL}^{-1}$

Figure 3. Mass spectra of MLu (A) Q1 (parent ion) scan with declustering potential 61 V, (B) product ion scan of m/z 1106.5 with collision potential 60 V (60 eV collision energy).

MLu was calculated by multiplying the $\mu\text{g mL}^{-1}$ concentration by a factor of 6.66.

RESULTS

LC-MS/MS

Method Development

Choice of IS and MS Parameters. The IS used for LC-MS/MS (PCI-0353) is a chemical analogue of MLu with an extra ethyl ether group in the side chain. The

chemical similarity enables the IS and the analyte to behave similarly during sample processing, chromatography, ionization at the interface of the LC/MS, fragmentation, and detection in the mass spectrometer. Two acetate counterions are associated with 1 molecule of MLu or the IS to form a complex structure (**Figure 1**).

The Q1 (parent) mass spectrum of MLu in **Figure 3A** showed 2 ions, m/z 1106.5 and 524.1, from MLu. The m/z 1106.5 ion is a singly charged ion, and the mass

matches the complex losing 1 acetate counterion. The ion with m/z 524.1 is a doubly charged ion based on 0.5 mass differences between isotope peaks (insert of **Figure 3A**), and it is an MLu^{2+} ion without both acetate counterions. The abundance ratio of m/z 524.1 to 1106.5 remained 5.5:1 with various declustering potentials up to 70 V, which indicates that the dissociation of acetate counterions happened in the liquid phase rather than in the gas phase. The remaining single Oac MLu (or IS) complex was very stable under collision-activated dissociation conditions and showed no fragment ions, including m/z 524.1 with collision potential up to 60 V (collision energy 60 eV for a singly charged ion) in the product ion spectrum (**Figure 3B**). The collision gas used was N_2 . The signal of parent ion m/z 1106.5 significantly dropped when applying collision energy above 60 eV and still showed no fragmentation. The product ion spectrum of m/z 524.1 showed the same pattern of no fragmentation. However, the ion signal disappeared when applying collision potential more than 30 V, which transferred 60 eV collision energy to the doubly charged ion of m/z 524.1.

Under the MRM of mass spectrometer, the parent and product ions of the compounds of interest retained the same m/z of 1106 and 1150 for MLu and the IS, respectively. With high collision potential (60 V), the background noise was minimized by fragmenting potential interfering ions to different product ions, resulting in a good signal-to-noise ratio for sensitivity and selectivity. The same strategy has been used in our laboratory in LC-MS/MS bioanalytical methods for hard-to-fragment analytes to minimize background noise.^{25,26} Chromatograms of MLu and the IS in extracted plasma solutions are shown in **Figure 4**. The compounds have the same retention time. The IS m/z was distinctly different from that of the analyte.

Sample Extraction. The Lu (III) metal ion positioned within the MLu macrocycle imparts a net 2+ charge to the molecule, which results in coordination with negatively charged counterions present in solution. Because of the polar nature of MLu , liquid/liquid extraction using an organic solvent was not attempted. Solid-phase extraction on an Oasis HLB cartridge was attempted using a buffered 50% acetonitrile mixture to elute the compounds, resulting in poor, inconsistent recovery and a noisy baseline in the LC-MS/MS chromatogram. Therefore, an acetonitrile protein precipitation method previously developed for the analysis of an analogous metallotexaphyrin was adapted for the quantitation of MLu . Acetonitrile was our first choice because an extract of aqueous acetonitrile solution could be directly injected onto the LC-MS/MS system and was also

compatible with the LC mobile phase. Plasma samples could have been precipitated with an acid, such as perchloric acid, but the resulting extract would not have been compatible with the LC-MS/MS and may have resulted in severe matrix suppression.

Acidification of the acetonitrile with acetic acid resulted in a good MS signal. Both ammonium acetate and acetic acid were tested in the precipitation mixture; better peak shape was seen with the acetic acid. MLu was known to adhere to glass, so polypropylene microfuge tubes were chosen for the sample preparation; they could be subjected to higher g forces during centrifugation, resulting in a cleaner supernatant. Recovery of MLu from plasma samples was determined by comparing the MLu mass spectrometer response for plasma samples that were spiked and then extracted with the MS response seen for samples that were first extracted and then spiked.

The results for recovery and matrix effect are shown in **Table 1**. With protein precipitation, MLu and the IS recoveries were high and consistent over the entire range of the method. The mean recovery was 86% for MLu and 87% for the IS. The coefficient of variation (CV) for the extracted samples was $\leq 8.0\%$ for MLu and 13.0% for the IS. Matrix enhancement was seen in the presence of plasma for MLu (7%-17%) and the IS (15%).

Chromatography. Since a sufficient and consistent signal was seen with simple protein precipitation, chromatographic resolution of analytes from interfering species in the matrix was subsequently addressed. Ionization of analytes could be suppressed by other competing ions from the sample matrix, especially when electrospray was used.²⁵⁻²⁹ It was important to provide separation of the analyte from interfering matrix components. Various columns were tested, and the analytical column selected provided adequate separation of the analyte from the majority of the interfering species in the matrix. Initially, a Keystone Inertsil ODS2 silica column (50 $\times$ 3 mm, 5 μ m) was used; however, interfering late-eluting matrix peaks were seen. Increasing the column size to 100 $\times$ 4.6 mm, 5 μ m bead size, did not increase the resolution of analyte peaks from interfering peaks. An isocratic system using a Zorbax Eclipse XDB-C18 column and mobile phase consisting of acetonitrile:ammonium acetate buffer (100 mM, pH 4.3) (60:40, vol/vol) was tried. This set of chromatographic conditions yielded acceptable separation of analyte peaks from interfering peaks. Most of the hydrophilic components in the plasma were eluted in the

Figure 4. Chromatograms of extracted MLu plasma sample: (A) MLu at 1.0 µg mL⁻¹, (B) IS at 0.1 µg mL⁻¹.

solvent front. However, the matrix effect was not completely eliminated. Although the major signal-suppressing components from the matrix were separated from the analyte, a slight but consistent matrix enhancement of the analyte response was observed.

Validation Performance

Sensitivity and Selectivity. The accuracy of the method was expressed as the percent relative error (RE), which was calculated as follows: (measured concentration-theoretical concentration)/theoretical concentration x 100. The precision was expressed as

the CV. **Table 2** shows lot-to-lot accuracy and precision data calculated from 10 independent lots of plasma. Also shown are the accuracy and precision data for a single lot of plasma spiked to a final concentration of 0.05 µg mL⁻¹ (Lower Limit of Quantitation [LLOQ]) and analyzed 6 times as part of a single analytical run. To test lot-to-lot matrix effect on sensitivity and selectivity, 10 individual lots were tested after spiking with the analyte at concentrations of 0, 0.05, and 1 µg mL⁻¹. No individual lot showed any interference at the retention times of the compounds of interest. The signal-to-noise ratio was at least 15:1 at the LLOQ. The CV and RE of the mean concentra-

Table 1. LC-MS/MS Method: Extraction Recovery and Matrix Effect*

	Mean Peak Area, CV (%)			
	0.05 µg mL ⁻¹ MLu	0.50 µg mL ⁻¹ MLu	5.0 µg mL ⁻¹ MLu	IS
A. neat	6235 (9.3)	51 933 (1.5)	611 608 (1.3)	43 088 (7.2)
B. recovery control	6651 (17.8)	59 094 (16.3)	688 388 (11.3)	49 448 (21.9)
C. extracted	5555 (8.0)	50 334 (6.1)	605 246 (2.1)	43 221 (13.0)
% recovery	84	85	88	87
% matrix effect	7	17	13	15

*CV indicates coefficient of variation; IS, internal standard; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MLu, motexafin lutetium. MLu (0.05, 0.5, and 5.00 µg mL⁻¹) and IS in human plasma samples were extracted by protein precipitation. For each analyte, the extraction recovery was calculated by comparing the peak area in samples spiked prior to processing (extracted) with equivalently treated samples spiked after processing (recovery control). The matrix effect was calculated by comparing the peak area of each analyte in samples spiked after processing (recovery control) to samples spiked into a matrix-free solution (neat). The mean (CV [%]) for 6 determinations (MLu) or 18 determinations (IS) was reported for each concentration tested. The percent recovery was calculated as C/B x 100. The percent matrix effect was calculated as (B/A – 1) x 100.

Table 2. LC-MS/MS Method: Sensitivity and Selectivity*

Lot No.	A				B	
	0.05 µg mL ⁻¹		1.0 µg mL ⁻¹		0.05 µg mL ⁻¹	
	Calc. Conc.	% Dev.	Calc. Conc.	% Dev.	Calc. Conc.	% Dev.
1	0.0493	–1.4	1.03	3.0	0.0468	–6.4
2	0.0478	–4.4	1.01	1.0	0.0493	–1.4
3	0.0473	–5.4	0.999	–0.1	0.0494	–1.2
4	0.0475	–5.0	1.03	3.0	0.0457	–8.6
5	0.0433	–13.4	1.04	4.0	0.0506	1.2
6	0.0476	–4.8	1.02	2.0	0.0480	–4.0
7	0.0450	–10.0	0.992	–0.8		
8	0.0441	–11.8	1.01	1.0		
9	0.0447	–10.6	0.976	–2.4		
10	0.0457	–8.6	0.994	–0.6		
Mean	0.0462		1.010		0.0483	
CV (%)	4.2		2.0		3.8	
RE (%)	–7.6		1.0		–3.4	
n	10		10		6	

*Calc. Conc. indicates calculated concentration; CV, coefficient of variation; Dev., deviation; LC-MS/MS, liquid chromatography–tandem mass spectrometry; RE, relative error. A: 10 independent human plasma lots, with each lot analyzed once spiked with MLu (0.05 and 1.0 µg mL⁻¹). B: Single spiked human plasma lot (0.05 µg mL⁻¹) analyzed as 6 replicates.

tion calculated from the 10 lots of plasma spiked at the LLOQ were 4.2% and –7.6%, respectively. Therefore, among the 10 matrix lots, there were no major differences that significantly affected the accurate quantification of MLu. The CV and RE of the mean concentration calculated from 6 replicate analyses of a single lot of plasma were 3.8% and –3.4%, respectively, which met the pharmaceutical industry's acceptance criteria of less than or equal to ±20% for bias and imprecision at the LLOQ.³⁰

Several plasma lots were pooled for preparation of standards and QC samples. Representative chromatograms of the LLOQ compared to the blank control plasma are shown in **Figure 5**. The signal-to-noise ratio at the LLOQ for the pooled lot used for standard preparation was 15:1. Processed spiked samples containing only the IS had an interfering peak detected as MLu in some of the assay curves. When present, the interference peak quantitated between 5% and 12.6%

Figure 5. Chromatograms of extracted plasma samples: (A) MLu at LLOQ concentration of $0.05 \mu\text{g mL}^{-1}$, (B) blank MLu control, and (C) blank IS control.

of the LLOQ. This magnitude of interference did not affect the accuracy of LLOQ quantification.

Linearity, Bias, and Precision. The standard curve was linear over the concentration range of 0.05 to 5.0

$\mu\text{g mL}^{-1}$. The mean coefficient of determination (r^2) $\pm$ SD calculated from 5 human plasma validation runs was 0.99954 ± 0.00025 ; the mean slope was $2.2922 \text{ mL } \mu\text{g}^{-1}$, with 2.3% CV. **Table 3** (A columns) shows the standard curve summary for these 5 validation

Table 3. Precision and Accuracy of Interday Calibration Standards*

Concentration Added ($\mu\text{g mL}^{-1}$ Lu)				Mean Concentration Found ($\mu\text{g mL}^{-1}$ Lu)				CV (%)				RE (%)			
A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0.05	0.50	0.10	0.10	0.046	0.488	0.0998	0.0987	6.3	1.5	1.4	0.9	-8.4	-2.4	-0.2	-1.3
0.10	1.00	0.20	0.20	0.106	0.994	0.200	0.203	3.3	0.5	2.8	2.1	5.5	-0.6	0.1	1.7
0.20	3.00	0.60	0.60	0.202	3.02	0.597	0.612	6.0	0.9	0.9	0.5	0.9	0.8	-0.6	2.0
0.50	10.0	2.00	2.00	0.495	10.2	2.01	2.00	4.4	1.2	0.6	1.3	-1.1	1.7	0.3	0.0
1.00	20.0	4.00	4.00	1.02	20.1	4.01	3.98	3.3	0.7	0.4	0.9	2.1	0.7	0.2	-0.5
2.00	30.0	6.00	6.00	2.04	30.2	5.98	6.07	1.4	0.6	0.7	2.0	2.2	0.7	-0.3	1.1
4.00	40.0	8.00	8.00	4.01	39.9	7.95	7.85	2.8	0.7	0.6	4.4	0.3	-0.2	-0.6	-1.9
5.00	50.0	10.0	10.0	4.93	49.5	10.1	9.86	2.0	0.7	2.0	1.0	-1.5	-1.0	0.9	-1.4

*CV indicates coefficient of variation; EDTA, ethylenediaminetetraacetic acid; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Lu, lutetium; RE, relative error. A: LC-MS/MS method. Standard curve results from five validation runs (concentration unit: $\mu\text{g mL}^{-1}$ MLu). B: ICP-AES method (high concentration range in EDTA plasma). Standard curve results from 6 validation runs. C: ICP-AES method (low concentration range in EDTA plasma). Standard curve results from 5 validation runs. D: ICP-AES method (low concentration range in heparinized whole blood). Standard curve results from five validation runs.

Table 4. Within-Run and Between-Run Precision and Accuracy for Quality Control Samples Prepared in Human Plasma*

Interday, n = 30 (n = 36 in B)															
Concentration Added ($\mu\text{g mL}^{-1}$ Lu)				Mean Concentration Found ($\mu\text{g mL}^{-1}$ Lu)				CV (%)				RE (%)			
A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0.15	1.50	0.30	0.30	0.152	1.56	0.316	0.307	6.3	8.7	1.5	2.8	1.3	4.3	5.3	2.2
0.75	15.0	3.00	3.00	0.766	14.6	3.16	3.09	3.3	1.3	1.0	1.4	2.2	-2.9	5.3	2.9
3.75	37.5	7.50	7.50	3.67	36.3	7.86	7.66	3.4	1.3	1.1	1.3	-2.1	-3.1	4.8	2.2
			25.0				25.6				2.7				2.6
Intraday, n = 6															
Concentration Added ($\mu\text{g mL}^{-1}$ Lu)				Mean Concentration Found ($\mu\text{g mL}^{-1}$ Lu)				CV (%)				RE (%)			
A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0.15	1.50	0.30	0.30	0.153	1.60	0.314	0.301	3.2	5.2	2.0	1.3	2.07	6.6	4.7	0.4
0.75	15.0	3.00	3.00	0.765	14.4	3.12	3.10	1.2	0.8	0.8	1.1	1.9	-4.1	4.0	3.3
3.75	37.5	7.50	7.50	3.62	36.2	7.78	7.78	3.2	1.5	0.5	0.5	-3.5	-3.5	3.7	3.7
			25.0				25.8				2.6				3.0

*CV indicates coefficient of variation; EDTA, ethylenediaminetetraacetic acid; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Lu, lutetium; RE, relative error. A: LC-MS/MS method (concentration unit: $\mu\text{g mL}^{-1}$ MLu). B: ICP-AES method (high concentration range in EDTA plasma). C: ICP-AES method (low concentration range in EDTA plasma). D: ICP-AES method (low concentration range in heparinized whole blood).

runs, which were performed on 2 LC-MS/MS systems, by 2 analysts, over 12 days. For the back-calculated values of all plasma standards at each concentration level, the interday CV was $\leq 6.3\%$ and the interday RE was within 8.4%. Six replicate injections

of plasma QC samples at each of 3 concentrations levels were also included in these 5 validation runs. As shown in **Table 4** (A columns), the interday CV was $\leq 6.3\%$ and the interday RE was within 2.2%; the

intraday CV was $\leq 3.2\%$ and the intraday RE was within 3.5%.

ICP-AES

Method Development

Signal Optimization. The emission line used for MLu is 1 of 4 primary lines available, of which the strongest was chosen. Interference, signal strength, and sensitivity were optimized when using this primary line. For In, only 2 primary lines were available. The second strongest line was chosen because it yielded the optimum condition for good sensitivity and minimal interference. Heparinized plasma, heparinized whole blood, and serum were also tested and observed to be free of interference.

Validation Performance

Linearity, Bias, and Precision. Two standard curve ranges were validated in EDTA human plasma to meet the expected range of sample concentrations from different clinical protocols: 0.5 to 50 and 0.1 to 10 $\mu\text{g Lu mL}^{-1}$. For 6 EDTA human plasma standard curves prepared over the high concentration range, the mean coefficient of determination (r^2) $\pm$ SD was 0.99995 ± 0.00006 and the mean slope was $0.1018 \text{ mL } \mu\text{g}^{-1}$, with 2.7% CV. The interday CV was $\leq 1.5\%$ and the interday RE was within 2.4% for the back-calculated values of all plasma standards at each concentration level (**Table 3**, B columns). For 5 human plasma standard curves prepared over the low concentration range, the mean coefficient of determination (r^2) $\pm$ SD was 0.99986 ± 0.00010 and the mean slope was $0.5764 \text{ mL } \mu\text{g}^{-1}$, with 1.0% CV. The interday CV was $\leq 2.8\%$ and the interday RE was within 0.9% for the mean back-calculated values of all plasma standards at each concentration level (**Table 3**, C columns).

For both the high concentration range and the low concentration range, a set of validation samples were prepared at the LLOQ and QC samples were prepared at 3 concentration levels spanning the linear range of the method. For the high concentration range LLOQ validation samples ($0.5 \mu\text{g mL}^{-1}$ Lu), the intraday CV was 3.7% and the intraday RE was 10.5%, $n = 14$. For all other QC samples in the high concentration range, the interday CV was $\leq 8.7\%$ and the interday RE was within 4.3%; the intraday CV was $\leq 5.2\%$ and the intraday RE was within 6.6% (**Table 4**, B columns). For the low concentration range LLOQ validation samples, the intraday CV was 4.9% and the RE was 4.9%. For all other QC samples in the low concentration range, the

interday CV was $\leq 1.5\%$ and the interday RE was within 5.3%; the intraday CV was $\leq 2.0\%$ and the intraday RE was within 4.7% (**Table 4**, C columns).

The ICP-AES method was also validated over the low concentration range in heparinized whole blood. For 5 validation batches analyzed over 3 days, the mean (CV) coefficient of determination (r^2) $\pm$ SD was 0.99964 ± 0.00036 and the mean slope was $0.5451 \text{ mL } \mu\text{g}^{-1}$, with 5.0% CV. For the mean back-calculated values of all plasma standards at each concentration level, the interday CV was $\leq 4.4\%$ and the interday RE was within 2.0% (**Table 3**, D columns). A set of QC samples was prepared at 3 concentration levels spanning the linear range of the method. These QC samples were also analysed as part of the validation. For the QC samples, the interday CV was $\leq 2.8\%$ and the interday RE was within 2.9%; the intraday CV was $\leq 1.3\%$ and the intraday RE was within 3.7%. In addition, a dilution QC sample was prepared at $25 \mu\text{g mL}^{-1}$ and diluted ($n = 6$ replicate dilutions) to fall within the linear range of the assay. The interday CV was 2.7% and the interday RE was 2.6%; the intraday CV was 2.6% and the intraday RE was 3.0% (**Table 4**, D columns).

The ICP-AES method was extended at the low concentration standard curve range ($0.1\text{--}10 \mu\text{g Lu mL}^{-1}$) to include heparinized plasma and serum in addition to EDTA plasma. For the cross-comparison, the QC samples were prepared in heparinized plasma and serum at the same 3 concentration levels in each matrix. The concentration for each QC sample was first back-calculated using a standard curve prepared in EDTA plasma, then back-calculated again using a matrix-matched standard curve. At each concentration level, the 2 sets of back-calculated values differed by less than 0.9%.

Analyte Stability

Stability of the MLu was a concern because preliminary data indicated that the compound was light-sensitive. Because MLu could generate reactive oxygen species upon exposure to visible light, it was important to protect samples from light after collection and during processing and storage. Therefore, the stability of MLu in plasma was tested before and after extraction using the LC-MS/MS method. When processed under either fluorescent or yellow light, MLu was stable in the plasma matrix for at least 4 hours at room temperature, 15 hours under refrigeration, and 23 months at $\leq 70^\circ\text{C}$ (**Table 5**). The plasma samples could be subjected to at least 6 cycles of freezing and thawing without the loss of MLu potency. After extraction, MLu

Table 5. Stability of MLu in Human Plasma and Processed Samples*

	LC-MS/MS		ICP-AES	
	Period	As Percentage of Control	Period	As Percentage of Control
In processed sample				
Reinjection	125 h	96-103	23 h	96-102
Refrigeration 2-8°C	57 h	96-97	72 h	98-102
On-system	4.7 h, 100 injections	99-103	Not done	Not done
In plasma sample				
Benchtop (under yellow and white lights, at room temperature)	4 h	93-103	27 h	99-101
In refrigerator 2-8°C	15 h	93-95	—	—
Freeze/thaw cycles	2 and 6 cycles	96-100	2 and 3 cycles	99-101
Long-term storage at -70°C	23 mo	100-101	39 wk	94-101

*ICP-AES indicates inductively coupled plasma-atomic emission spectroscopy; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MLu, motexafin lutetium.

was stable on the autosampler tray for 125 hours in the reconstitution solvent, as determined by reinjection stability. The extracted samples could also be stored in a refrigerator for at least 57 hours before injection.

Method Robustness

For some of the validation runs, a second LC-MS/MS system (Applied Biosystems/MDS Sciex API 365 platform and associated HPLC system) was used to ensure method ruggedness on multiple platforms. During the validation, 2 analytical columns were tested between the 2 systems, resulting in similar chromatographic performance, with a CV of $\leq 5.0\%$ and an RE of $\leq 6.5\%$ for all batches. In subsequent clinical sample analysis, 4 additional columns were used with consistent chromatographic performance. Three different analysts performed extractions during the validation. Results of these extractions yielded a CV of $\leq 4.0\%$ and an RE of $\leq 6.0\%$ among analysts. Since the validation, 8 additional analysts have been validated to assay clinical samples.

Application to Clinical Sample Analysis

A pharmacokinetic study of photoangioplasty in subjects with peripheral arterial disease was conducted in 47 individuals (34 males and 13 females). In this clinical safety trial, subjects received Antrin (MLu) injection as a single intravenous infusion (1-5 mg kg⁻¹). Plasma or serum samples were obtained at baseline and

at 2 to 11 serial time points obtained over ≥ 6 days after dosing.

MLu and total elemental lutetium concentrations in plasma were measured using LC-MS/MS and ICP-AES, respectively. To facilitate comparison with the LC-MS/MS data, elemental lutetium concentrations (determined by ICP-AES) were converted to equivalent concentrations of MLu before pharmacokinetic analysis.

Figure 6 shows concentration-time data derived from a representative patient enrolled in the trial. This patient received a single intravenous 10-minute infusion of 3 mg kg⁻¹ MLu. As shown in **Figure 6**, both methods yielded similar values for C_{max} (35.1 $\mu\text{g-equiv mL}^{-1}$ MLu and 33.4 $\mu\text{g mL}^{-1}$ MLu for ICP-AES and LC-MS/MS, respectively), indicating that 95% of the total elemental lutetium in the plasma sample corresponding to t_{max} could be attributed to MLu. As indicated in **Figure 7**, this percentage decreased to 59% at 48 hours postdosing, suggesting that degradation products and/or metabolites of MLu may exist in plasma samples obtained at later time points. These degradation products/metabolites could result from slow degradation/metabolism of the compound in plasma/tissue, enterohepatic uptake of elemental lutetium following biliary elimination, and/or a tissue compartment associated with slow elimination of elemental lutetium. However, because of the rapid initial clearance of the drug from plasma, lutetium derived from species other than MLu did not make a major contribution to the area under the curve (AUC). Using noncompartmental

Figure 6. Plasma concentration-time profiles of MLu for Patient 0212, who received a single 3 mg kg⁻¹ intravenous administration of MLu. Open circles represent concentrations derived from ICP-AES measurements of elemental lutetium, which were converted to equivalent concentrations of MLu to facilitate comparison. Solid triangles represent concentrations of MLu measured by LC-MS/MS.

Figure 7. The percentage of total elemental lutetium in plasma (measured by ICP) attributed to MLu (measured by LC-MS/MS) versus time postdosing for Patient 0212. The solid line represents a weighted nonlinear regression of the data to a biexponential equation.

analysis, $AUC_{0-48 \text{ hour}}$ was calculated based on plasma MLu concentration-time data (LC-MS/MS) and found to equal $183.9 \mu\text{g}\cdot\text{hr mL}^{-1}$. The result was divided by the corresponding value calculated using ICP-AES-derived elemental lutetium concentration-time data, after converting lutetium concentrations to equivalent concentrations of MLu ($255.7 \mu\text{g-equiv}\cdot\text{hr mL}^{-1}$). This ratio, expressed as a percentage, revealed that during the 48-hour period following administration, 72% of the total systemic exposure to lutetium-containing species could be attributed to MLu.

CONCLUSION

LC-MS/MS and ICP-AES methods were developed and validated for the determination of MLu and elemental lutetium, respectively, in human plasma. These assays require small amounts of plasma, use a simple sample preparation procedure, and have rapid run times. A full validation of both methods revealed that the assays were sensitive, accurate, rugged, and reproducible. In addition, trending analysis revealed that accuracy and reproducibility were maintained throughout months of sample analyses. The utility of the LC-MS/MS and ICP-AES methods was demonstrated by using both assays to analyze each plasma sample from a single representative patient. The pharmacokinetic data indicated some degradation/metabolism of MLu in humans, but the majority of elemental lutetium in plasma was attributed to that contained in intact MLu.

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REFERENCES

1. Sessler JL, Murai T, Lynch V, Cyr M. An "expanded porphyrin": the synthesis and structure of a new aromatic pentadentate ligand. *J Am Chem Soc.* 1988;110:5586-5588.
2. Mody TD, Fu L, Sessler JL. Texaphyrins: synthesis and development of a novel class of therapeutic agents. In: Karlin KD, ed. *Progress in Inorganic Chemistry*. New York, NY: John Wiley & Sons; 2001:551-598.
3. Sessler JL, Mody TD, Hemmi GW, Lynch V. Synthesis and structural characterization of lanthanide(III) texaphyrins. *Inorg Chem.* 1993;32:3175-3187.
4. Sessler JL, Hemmi G, Mody TD, Murai T, Burrell A, Young S. Texaphyrins: synthesis and applications. *Acc Chem Res.* 1994;27:43-50.
5. Mody TD, Sessler JL. Porphyrin- and expanded porphyrin-based diagnostic and therapeutic agents. In: Reinhoudt DN, ed. *Supramolecular Technology*. New York, NY: John Wiley & Sons; 1999:245-294.
6. Young SW, Woodburn KW, Wright M, et al. Lutetium texaphyrin (PCI-0123): a near-infrared, water-soluble photosensitizer. *Photochem Photobiol.* 1996;63:892-897.
7. Guldi DM, Mody TD, Gerasimchuk N, Magda D, Sessler JL. Influence of large metal cations on the photophysical properties of texaphyrin, a rigid aromatic chromophore. *Am Chem Soc.* 2000;122:8289-8298.
8. Grossweiner LI, Bilgin MD, Berdusis P, Mody TD. Singlet oxygen generation by metallotexaphyrins. *Photochem Photobiol.* 1999;70:138-145.
9. Sessler JL, Dow WC, O'Connor D, et al. Biomedical applications of lanthanide (III) texaphyrins: Lutetium (III) texaphyrins as potential photodynamic therapy photosensitizers. *J Alloys Compounds.* 1997;249:146-152.
10. Harriman A, Maiya BG, Murai T, Hemmi G, Sessler JL, Mallouk TE. Metallotexaphyrins: a new family of photosensitizers for efficient generation of singlet oxygen. *J Chem Soc Chem Commun.* 1989;5:314-316.
11. Rockson SG, Lorenz DP, Cheong W, Woodburn KW. Photoangioplasty: an emerging clinical cardiovascular role for photodynamic therapy. *Circulation.* 2000;102:591-596.
12. Lum B, Wieman JT, Taber S, et al. Pharmacokinetics of lutetium texaphyrin (Lu-Tex) in a phase I trial of photodynamic therapy (PDT) of cancer (Clinical Pharmacology). *Proc Annu Meet Am Soc Clin Oncol.* 1997;16:243A.
13. Renschler MF, Yuen A, Panella TJ, et al. Photodynamic therapy trials with lutetium texaphyrin (Lu-Tex) in patients with locally recurrent breast cancer. *Proc Optimal Methods Tumor Treatment Detect: Mech Tech Photodynam Ther VII. SPIE.* 1998;3247:35-39.
14. Wieman TJ, Panella T, Lustig R, et al. Photodynamic therapy (PDT) of locally recurrent breast cancer (LRBC) with lutetium texaphyrin (Lutrin): a phase IB/IIA trial. *Proc ASCO.* 1999;18:111a.
15. Kereiakes DJ, Szyniszewski AM, Simon DI, et al. Coronary photoangioplasty using motexafin lutetium (Antrin®): early findings of a phase I safety trial in patients undergoing stent implantation. *Circulation.* 2000;102:565-565.
16. Rockson SG, Kramer P, Razavi M, et al. Photoangioplasty for human peripheral atherosclerosis: results of a phase I trial of photodynamic therapy with motexafin lutetium (Antrin). *Circulation.* 2000;102:2322-2324.
17. Sessler JL, Miller RA. Texaphyrins: new drugs with diverse clinical applications in radiation and photodynamic therapy. *Biochem Pharmacol.* 2000;59:733-739.
18. Woodburn KW, Fan Q, Kessel D, Luo Y, Young SW. Photodynamic therapy of B16F10 murine melanoma with lutetium texaphyrin. *J Invest Dermatol.* 1998;110:746-751.
19. Woodburn K, Qing F, Kessel D, Young S. Photoeradication and imaging of atheromatous plaque with texaphyrins. *Proc Lasers Surg: Adv Characteriz, Ther, Sys VII. SPIE.* 1997;2970:44-50.

20. Woodburn KW, Young SW, Fan Q, Kessel D, Miller RA. Selective uptake of texaphyrins by atheromatous plaque. *Proc Lasers Surg: Adv Characteriz, Ther, Sys VI*. SPIE. 1996;2671:62-71.
21. Woodburn KW, Fan Q, Miles DR, Kessel D, Luo Y, Young SW. Localization and efficacy analysis of the phototherapeutic lutetium texaphyrin (PCI-0123) in the murine EMT6 sarcoma model. *Photochem Photobiol*. 1997;65:410-415.
22. Parise RA, Miles DR, Egorin MJ. Sensitive high-performance liquid chromatographic assay for motexafin gadolinium and motexafin lutetium in human plasma. *J Chromatogr*. 2000;749:145-152.
23. Normann PT, Joffe P, Martinsen I, Thomsen HS. Quantification of gadodiamide as Gd in serum, peritoneal dialysate and faeces by inductively coupled plasma atomic emission spectroscopy and comparative analysis by high-performance liquid chromatography. *J Pharm Biomed Anal*. 2000;22:939-947.
24. Sessler JL, Tvermoes NA, Guldi DM, Mody TD, Allen WE. One-electron reduction and oxidation studies of the radiation sensitizer gadolinium(III) texaphyrin (PCI-0120) and other water soluble metallotexaphyrins. *J Phys Chem*. 1999;103:787-794.
25. Nachi R, Weng N, Lee JW, Hulse JD, Lin PP. Method improvements for LC/MS/MS bioanalysis of buprenorphine in human plasma. *AAPS PharmSci*. 1999; 1(suppl):S-303. Abstract 2809. Available at: http://www.aapspharmsci.org/abstracts/AM_1999/1140.htm.
26. Jiang X, Sartwell C, Petersen M, et al. A sensitive LC/MS/MS method for the quantitation of cyclosporin A in human blood. In: *Proceedings the 46th ASMS Conference on Mass Spectrometry and Allied Topics*. 1998:1459. Available at: <http://www.inmerge.com/aspfolder/ASMSAbstracts.html>, using 'cyclosporin' in text search.
27. Kebarle P, Tang L. From ions in solution to ions in the gas phase: the mechanism of electrospray mass spectrometry. *Anal Chem*. 1993;65:972A-986A.
28. Matuszewski BK, Constanzer ML, Charez-Eng CM. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per millilitre concentrations. *Anal Chem*. 1998;70:882-889.
29. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. Mechanistic investigation of ionisation suppression in electrospray ionisation. *J Am Soc Mass Spectrom*. 2000;11:942-950.
30. Shah VP, Midha KK, Dighe S, et al. Analytical methods validation: bioavailability, bioequivalence, and pharmacokinetic studies. *J Pharm Sci*. 1992;81:309-312.