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LC-MS/MS determination of a farnesyl transferase inhibitor in human plasma and urine

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Abstract

To support clinical pharmacokinetic studies in cancer patients, sensitive and specific methods for measuring 4-[1-(4-cyanobenzyl)-5-imidazolylmethyl]-1-(3-chlorophenyl) piperazinone (I), a farnesyl transferase inhibitor (FTI), in human plasma and urine were developed and validated. The methods are based on high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization (APCI) and tandem mass spectrometric (MS/MS) detection in the positive ion mode using a heated nebulizer interface. Drug and internal standard were isolated from plasma or basified urine using automated solid-phase extraction on cyano cartridges. The organic extracts were dried, reconstituted in aqueous acetonitrile and injected into the system. Chromatographic separation of **I** and internal standard (**IS**) was achieved using a BDS Hypersil C8 analytical column, with a mobile phase consisting of acetonitrile:methanol:water (50:4:46) and trifluoroacetic acid (0.05%) at a flow rate of 0.6 ml/min. MS/MS detection was performed on a PE-Sciex API 300 tandem mass spectrometer operated in selected reaction monitoring mode. The parent → product ions monitored were m/z 406 → 195 for analyte **I** and m/z 448 → 195 for the internal standard. Unusual in this method is that quantitation is accomplished using a secondary product ion, m/z 195, of drug I and IS. The assays were validated over the concentration range of 0.5–1000 ng/ml (1.2 nM to 2.5 μM, respectively) in plasma, and 2.5–500 ng/ml (6.2 nM to 1.23 μM) in urine. Accuracy was within ±10% of nominal concentration at all levels in urine, and all but the lowest standard in plasma (±14% at 0.5 ng/ml). Intraday precision (expressed as coefficients of variation, CVs) for standard replicates and interday precision for quality control (QC) samples were less than 8% at all concentrations in both matrices. Detailed descriptions of the extraction procedure and analytical methodology used in the assay of **I** in plasma and urine are presented. This procedure may have utility in the quantitation of other imidazole-based FTIs with cyanobenzyl substructures.

Keywords: Liquid chromatographic-tandem mass spectrometric assay; HPLC-MS/MS; Farnesyl transferase inhibitor; FTI

1. Introduction

Methodology is described herein for quantitation of a farnesyl transferase inhibitor (FTI) in plasma and urine. The importance of this metho-

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dology is related to the progress and development of FTIs as chemotherapeutics.

Mutant Ras proteins are associated with many types of human tumors. Naturally- occurring mutations lock the Ras protein in its active form and result in an uninterrupted growth signal [1]. Ras proteins are post-translationally farnesylated by the enzyme farnesyl transferase (FT). Farnesylation is important as it promotes membrane binding [2] and a complicated series of steps that propagate signals from the plasma membrane to the nucleus promoting cell proliferation and differentiation.

A number of pharmaceutical companies have investigated FTIs in clinical trials, but over the last few years scientists have questioned the theory of how FTIs work to inhibit tumor growth. Originally, inhibition of farnesylation of Ras was thought to be the only mechanism of inhibition of tumor growth by FTIs [3,4]. More recently, research has shown that upon inhibition of FT, two Ras proteins (N-Ras and K-Ras, found in 90% of tumors) become substrates for another enzyme, geranylgeranyltransferase (GGT). Activation with GGT results in geranylgeranylated Ras proteins that appear to be functionally equivalent to farnesylated proteins. Additionally, other substrates besides Ras are farnesylated by FT that may be involved in Ras transformation and tumor growth [3–5].

With the initial goal of inhibiting the transforming activity of K-Ras, **I** (Fig. 1) a potent inhibitor of FT ($IC_{50} = 2$ mM) and GGT ($IC_{50} = 98$ μM) containing a cyanobenzylimidazole substructure was developed [1]. More than half of the compounds reviewed in the patent literature for FTI [3,4] contain a similar chemical structure. Currently, several pharmaceutical companies are developing combination treatments for cancer using an FTI and other known chemotherapeutic agents [3,4]. To date, no sensitive HPLC-MS/MS methods have been reported for this important class of compounds for cancer chemotherapy.

Sensitive and specific methods were developed and validated for the assay of **I** in human plasma and urine to support clinical studies in subjects with malignancies. These methods are relevant to quantitation of other FTIs of similar chemical structure. **I** and an internal standard (IS, Fig. 1) were isolated from plasma or urine by automated solid-phase extraction (SPE) on cyano (CN) cartridges using a Gilson Aspec XL system. Reverse-phase chromatography was performed using a BDS Hypersil C8 HPLC column. Mass detection was accomplished using APCI and multiple reaction monitoring in the positive ion mode. Method validation data are presented for both plasma and urine. Plasma profiles of **I** in subjects from the initial Phase I study are presented to show the utility of the method for clinical studies.

2. Experimental

2.1. Chemicals and reagents

Drug **I** and **IS** were synthesized in-house (Merck Research Laboratories, Rahway, NJ) and used as received. Drug-free heparinized human control plasma was purchased from SeraTec Biologicals (New Brunswick, NJ). Human control urine was obtained in-house (Merck Research Laboratories, West Point, PA) from staff members. Control plasma and urine were stored frozen at −20 °C until used.

HPLC grade dibasic sodium phosphate and phosphoric acid, 85%, and Optima grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Spectrophotometric grade trifluoroacetic acid was obtained from Aldrich (Milwaukee, WI). Water was purified by Milli-Q from Millipore (Bedford, MA).

Fig. 1. Positive product ion mass spectra (A) or protonated drug **I** (*m/z* 406) and (B) **IS** (*m/z* 488) with proposed chemical fragmentation pathways.

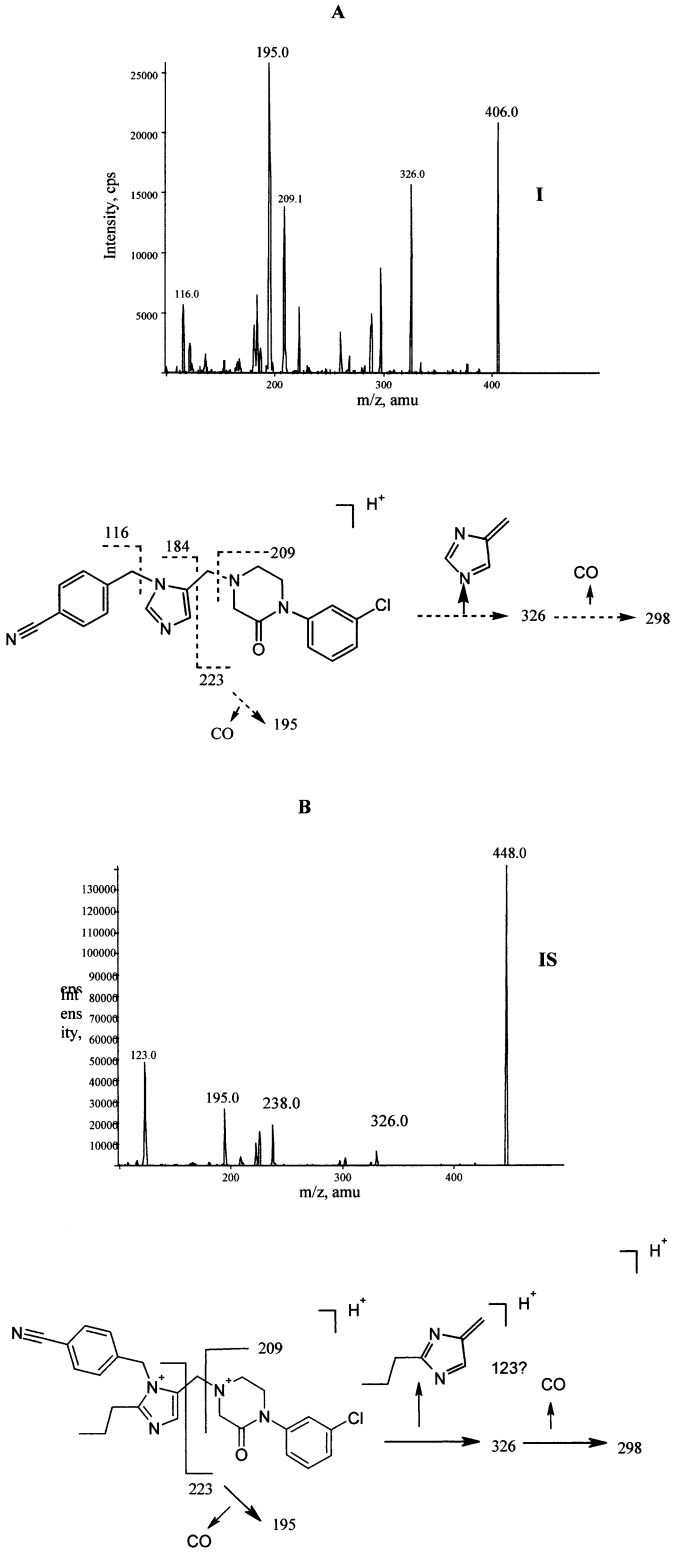
A
195.0
406.0
326.0
209.1
116.0
I
Intensity, cps
m/z, amu
H+
116
184
209
223
CO
195
326
298
B
448.0
IS
123.0
195.0
238.0
326.0
m/z, amu
209
123?
223
CO
195
326
298

Fig. 1

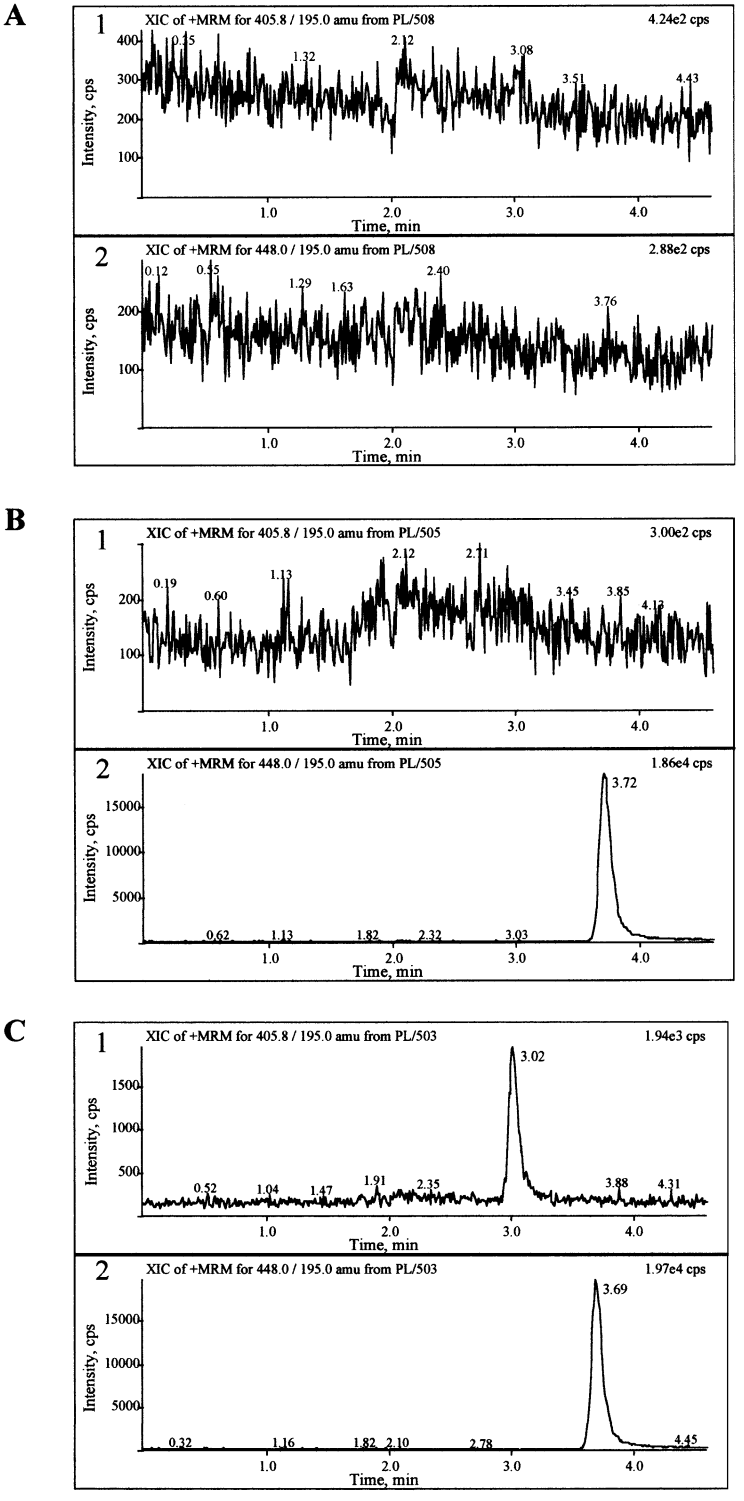
A
1
XIC of +MRM for 405.8 / 195.0 amu from PL/508
4.24e2 cps
Intensity, cps
Time, min
2
XIC of +MRM for 448.0 / 195.0 amu from PL/508
2.88e2 cps
Intensity, cps
Time, min
B
1
XIC of +MRM for 405.8 / 195.0 amu from PL/505
3.00e2 cps
Intensity, cps
Time, min
2
XIC of +MRM for 448.0 / 195.0 amu from PL/505
1.86e4 cps
Intensity, cps
Time, min
C
1
XIC of +MRM for 405.8 / 195.0 amu from PL/503
1.94e3 cps
Intensity, cps
Time, min
2
XIC of +MRM for 448.0 / 195.0 amu from PL/503
1.97e4 cps
Intensity, cps
Time, min

Fig. 2

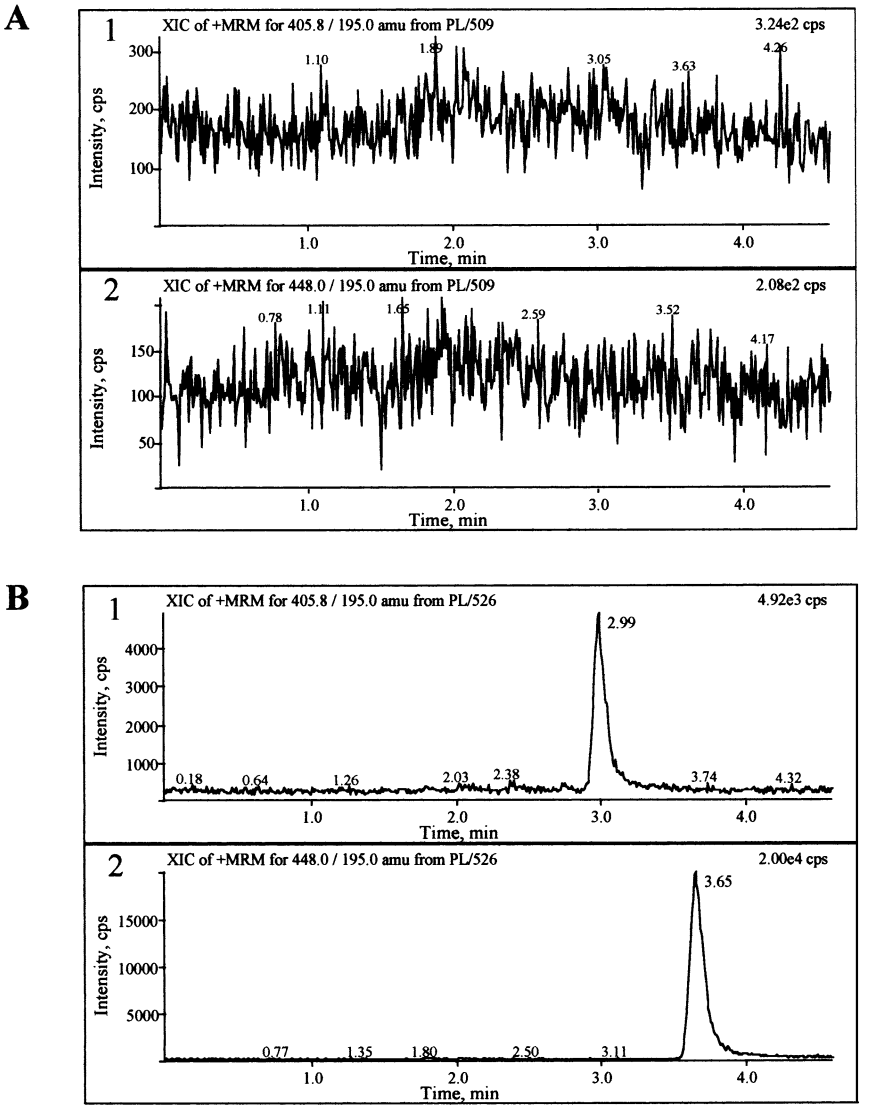

Fig. 3. Representative ion chromatograms of extracts of subject plasma samples obtained by multiple reaction monitoring: (A) predose plasma sample; (B) postdose plasma sample containing 10 ng/ml of **IS** (assay = 2.63 ng/ml of **I**). 1, channel for drug **I** (*m/z* 406 → 195); 2, channel for internal standards **IS** (*m/z* 448 → 195).

For use as standard solvent and reconstitution solvent, 50% acetonitrile (ACN) in water was prepared. Reagents for the extraction procedure were 20 and 80% methanol (MeOH) in water. A 1 M solution of dibasic sodium phosphate (Na_2HPO_4) was adjusted to pH 8 with phosphoric acid and was used to adjust the pH of urine samples prior to extraction.

Fig. 2. Representative ion chromatograms of control plasma extracts obtained by multiple reaction monitoring: (A) control plasma blank; (B) control plasma blank containing 10 ng/ml of **IS**; (C) standard in control plasma containing 1 ng/ml of **I** and 10 ng/ml of **IS**. 1, channel for drug **I** (*m*/z 406 → 195); 2, channel for internal standard **IS** (*m/z* 448 → 195).

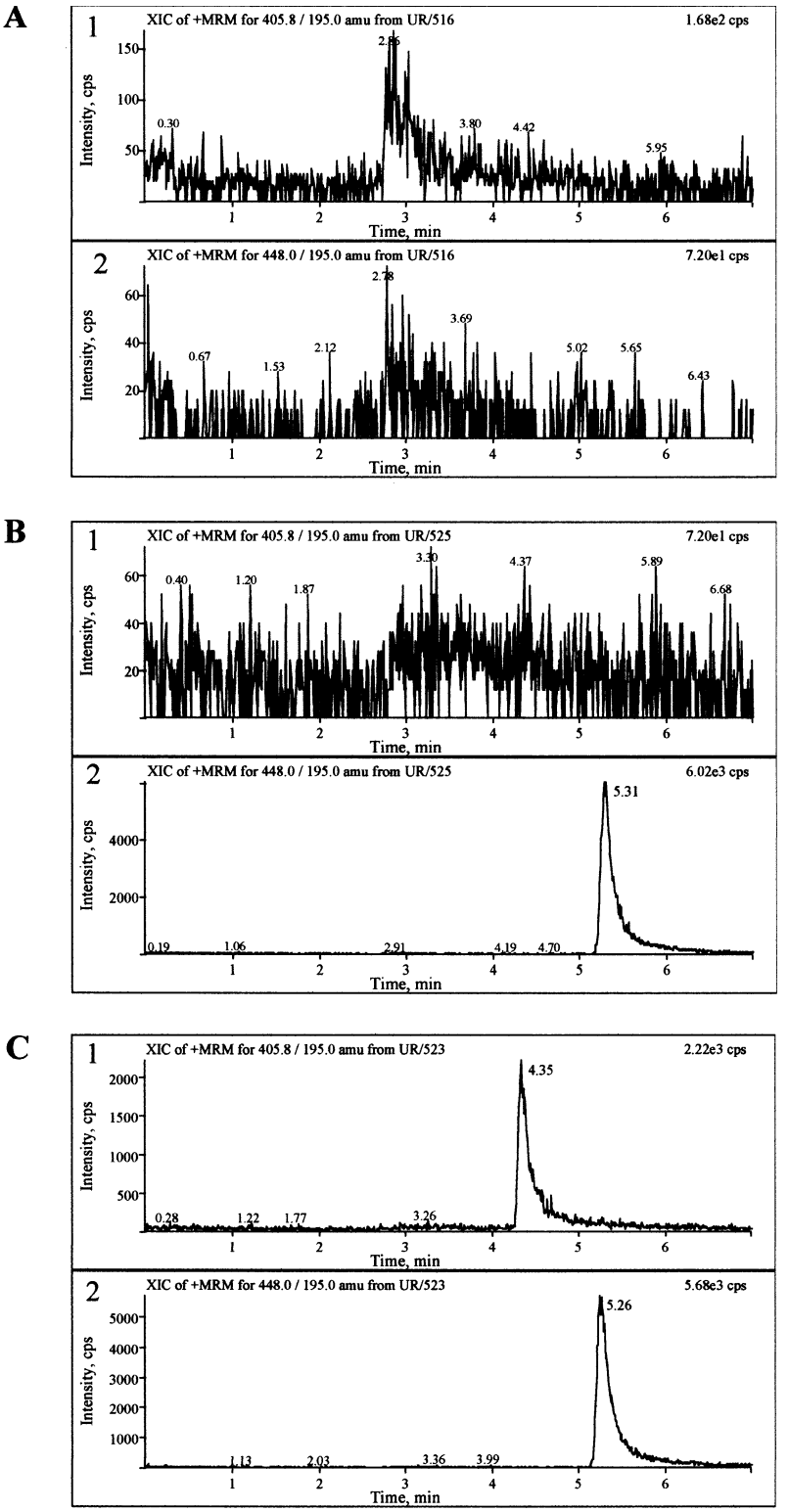
A
1
XIC of +MRM for 405.8 / 195.0 amu from UR/516
1.68e2 cps
Intensity, cps
Time, min
2
XIC of +MRM for 448.0 / 195.0 amu from UR/516
7.20e1 cps
B
1
XIC of +MRM for 405.8 / 195.0 amu from UR/525
7.20e1 cps
2
XIC of +MRM for 448.0 / 195.0 amu from UR/525
6.02e3 cps
C
1
XIC of +MRM for 405.8 / 195.0 amu from UR/523
2.22e3 cps
2
XIC of +MRM for 448.0 / 195.0 amu from UR/523
5.68e3 cps

Fig. 4

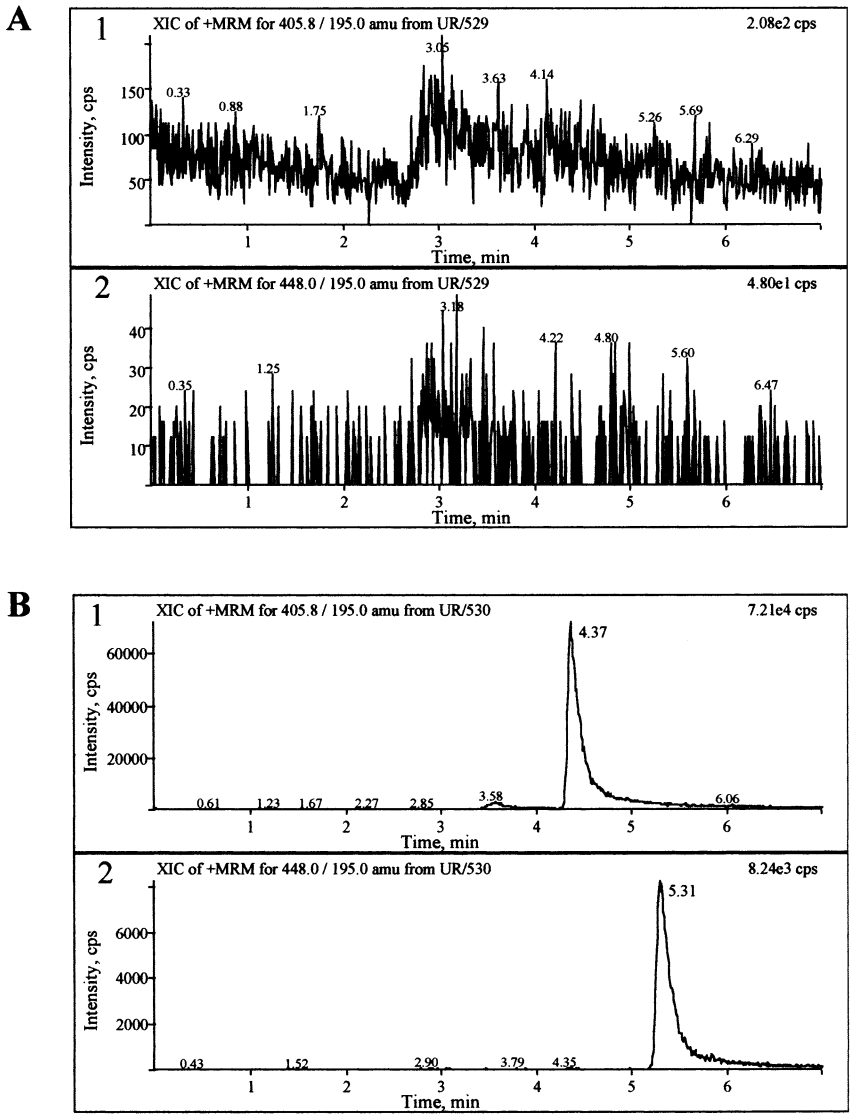

Fig. 5. Representative ion chromatograms of extracts of subject urine samples obtained by multiple reaction monitoring: (A) postdose urine sample; (B) postdose urine sample containing 10 ng/ml of **IS** (assay 116.4 ng/ml of **I**). 1, channel for drug **I** (m/z 406 → 195); 2, channel for internal standard **IS** (m/z 448 → 195).

2.2. Instrumentation

Liquid chromatography was performed using a Hewlett-Packard (Wilmington, DE) Series 1050 HPLC system consisting of an autosampler equipped with a 100-μl loop and a quaternary pump. The HPLC was coupled to a PE-Sciex (Foster City, CA) API 300 triple quadrupole mass

Fig. 4. Representative ion chromatograms of control urine extracts obtained by multiple reaction monitoring: (A) control blank urine; (B) control urine blank containing 10 ng/ml of **IS**; (C) standard in control urine containing 5 ng/ml of **I** and 10 ng/ml of **IS**. 1, channel for drug **I** (m/z 406 → 195); 2, channel for internal standard **IS** (m/z 448 → 195).

Table 1
Intraday accuracy and precision for plasma standard replicates

Nominal conc. (ng/ml)	n	Mean calculated conc.[a] (ng/ml)	Mean accuracy[a] (calculated/nominal, %)	Precision (CV, %)[b]
Standard				
0.5	6	0.572	114.4	7.78
1	6	0.970	97.0	4.79
2	6	1.93	96.7	4.85
5	6	4.82	96.4	4.35
10	6	9.54	95.4	2.22
20	6	19.83	99.2	3.24
50	6	50.10	100.2	2.96
100	6	100.7	100.7	3.52
250	5	265.0	106.0	5.25
500	5	499.3	99.8	5.40
1000	5	971.0	97.1	1.84

[a] Mean calculated concentration and accuracy derived from least squares linear regression: $1/x^2$ weighting of concentration vs. peak area ratio of $n = 63$ points (slope = 0.068, intercept = 0.003, correlation coefficient = 0.998).
[b] Coefficients of variation, expressed as percent, for standard replicates are based on peak area ratios [drug (**I**)/internal standard (**IS**) × 100].

spectrometer with an atmospheric pressure chemical ionization (APCI) interface. Data acquisition and processing were accomplished using Sciex MacQuan version 1.1 software.

Automated solid-phase extraction (SPE) of plasma and urine was carried out using an Aspec XL system from Gilson (Middleton, WI) and 1-ml isolute CN (EC) SPE cartridges, 100 mg sorbent, purchased from Jones Chromatography (Lakewood, CO).

2.3. Mass spec and chromatographic conditions

The Sciex API 300 was used with an APCI interface that was heated to 450 °C. The nebulizing gas (nitrogen, N_2) pressure was 80 psi and flow was approximately 8000 cc/min. The orifice and ring potentials were +44 and +200 V, respectively. The collision gas was N_2 (1.77×10^{15} molecules/cm^2). Multiple reaction monitoring (MRM) was in the positive ion mode. Parent and fragment ions monitored were m/z 406 → 195 for **I** and m/z 448 → 195 for **IS** using a 250-ms dwell time for each transition. Peak area ratios of drug **I** to **IS** were used to generate response for calibration curves and to calculate sample results.

For the plasma and urine assays the mobile phase consisted of 50% ACN, 46% of 0.05% TFA and 4% MeOH. The flow rate was 0.6 ml/min through a BDS Hypersil C8 analytical column from Keystone Scientific (Bellefonte, PA). For the plasma assay, the HPLC column was 100 × 4.6 mm with 5-μm particle size; for urine, 150 × 4.6 mm, 3 μm. For the plasma assay preceding the analytical column was an in-line prefilter containing a 2-μm stainless steel frit from Keystone Scientific. Chromatography was performed at room temperature using a 50-μl injection volume, and run times of 4 min for plasma samples and 7 min for urine samples.

2.4. Preparation of standards

A stock solution of **I** at 1 mg/ml as free base was prepared in ACN. For use in plasma calibration standards, working standards of 0.005–10 μg/ml of **I** were prepared by diluting the stock solution with 50% ACN in water. Similar dilutions of the stock were prepared in 50% ACN to yield working standards containing 0.025–5 μg/ml of **I** for use in urine calibration standards. All standards were stored at −20 °C between uses.

Table 2
Accuracy and precision for plasma quality controls

	1 ng/ml QC	50 ng/ml QC	500 ng/ml QC
Intraday statistics			
Mean calculated conc.[a]	0.97	52.0	486.6
S.D.	0.06	1.18	21.86
CV (%)[b]	6.29	2.27	4.49
% of nominal concentration	96.7	104.0	97.4
Interday statistics			
Mean calculated conc.[c]	1.03	52.0	493.4
S.D.	0.07	2.14	25.27
CV (%)	7.20	4.12	5.12
% of nominal concentration	103.3	104.0	98.7

[a] Mean calculated concentration of QCs ($n = 5$) calculated from least squares linear regression: $1/x^2$ weighting of concentration vs. peak area ratio.
[b] Precision represented as % coefficient of variation, $n = 5$.
[c] Mean calculated concentration of QCs ($n = 22$) calculated from least squares linear regression: $1/x^2$ weighting of concentration vs. peak area ratio.

IS stock solution at 10 μg/ml, as free base, was prepared in ACN. Subsequent dilutions of this stock were prepared on each day of use in 50% ACN to yield a 0.1 μg/ml working **IS** solution.

Plasma or urine calibration standards were prepared by adding 100 μl of each appropriate working standard and 100 μl of working **IS** to 1.0-ml aliquots of human control plasma or urine. The concentration range for the plasma calibration curve containing 11 standards was 0.5–1000 ng/ml. For urine, the calibration curve contained eight standards ranging from 2.5 to 500 ng/ml.

2.5. Preparation of quality control samples

For quality control (QC) samples, a 1-mg/ml primary stock solution of **I** was prepared in ACN in the same manner as for standard solutions, but from a separate weighing. The primary QC stock solution was diluted in 50% ACN in water to yield additional stock solutions of 100, 10, 1 and 0.1 μg/ml of **I**.

Plasma QC samples were prepared at three different concentrations. For high QCs (500 ng/ml), 250 μl of 100-μg/ml QC stock solution were diluted to 50 ml with control plasma. Similar dilutions of the 10- and 0.1-μg/ml QC stock solutions were prepared in control plasma to yield medium (50 ng/ml) and low (1 ng/ml) QCs, respectively. Plasma QC solutions were divided into 1.2-ml aliquots in polypropylene tubes and stored at −20 °C.

Urine QCs at 250 and 5 ng/ml were prepared and stored in a similar manner using control urine.

Table 3
Intraday accuracy and precision for urine standard replicates

Nominal conc. (ng/ml)	n	Mean calculated conc.[a] (ng/ml)	Mean accuracy[a] (calculated/nominal, %)	Precision (CV, %)[b]
Standard				
2.5	5	2.26	90.4	5.81
5	5	4.87	97.4	4.79
10	5	10.09	100.9	4.18
25	5	25.81	103.2	0.63
50	5	51.63	103.3	1.96
100	5	103.7	103.7	3.57
250	5	261.2	104.5	2.85
500	5	482.9	96.6	1.77

[a] Mean calculated concentration and accuracy derived from least squares linear regression: $1/x$ weighting of concentration vs. peak area ratio of $n = 40$ points (slope = 0.083, intercept = 0.051, correlation coefficient = 0.999).
[b] Coefficients of variation, expressed as percent, for standard replicates are based on peak area ratios [drug (**I**)/internal standard (**IS**) × 100].

Table 4
Accuracy and precision for urine quality controls

	5 ng/ml QC	250 ng/ml QC
Intraday statistics		
Mean calculated conc.[a]	4.74	236.8
S.D.	0.12	2.76
CV (%)[b]	2.61	1.17
% of nominal concentration	94.7	94.7
Interday statistics		
Mean calculated conc.[c]	4.57	250.7
S.D.	0.32	13.2
CV (%)[d]	7.04	5.27
% of nominal concentration	91.5	100.3

[a] Mean calculated concentration of QCs ($n = 5$) calculated from least squares linear regression: $1/x$ weighting of concentration vs. peak area ratio.
[b] Precision represented as % coefficient of variation, $n = 5$.
[c] Mean calculated concentration of QCs ($n = 10$) calculated from least squares linear regression: $1/x$ weighting of concentration vs. peak area ratio.
[d] Precision represented as % coefficient of variation, $n = 10$.

2.6. Extraction procedure

Control plasma, QCs and clinical samples were thawed at room temperature, mixed by vortex and centrifuged at $2000 \times g$ for 10 min in order to sediment the fibrin. Aliquots of 1.0 ml of control plasma, QCs or plasma samples were added to glass culture tubes and mixed with the following: 100 μl of 0.1-μg/ml working **IS** and 100 μl of **I** working standard or 50% ACN in water. The culture tubes were mixed by vortex for 30 s.

Using the Gilson Aspec XL, CN (EC) cartridges were conditioned twice with 1 ml MeOH, then washed twice with 1 ml water. A 1.2-ml volume of each plasma sample was loaded onto a cartridge, and washed once with 1 ml water and twice with 1 ml 20% MeOH in water. Samples were eluted into glass culture tubes using 1 ml 80% MeOH in water. Using a Zymark (Hopkinton, MA) TurboVap LV, the eluent was evaporated under a stream of air in a 50 °C water bath. Samples were then reconstituted in 200 μl of 50% ACN in water and mixed by vortexing and sonicating, and a 50-μl aliquot was injected into the HPLC-MS/MS for analysis.

Urine QCs and clinical samples were thawed at room temperature. Aliquots of 1.0 ml of control urine, QCs or urine samples were added to glass culture tubes and combined with the following: 100 μl of 0.1-μg/ml working **IS**, 100 μl of **I** working standard or 50% ACN in water, and 100 μl of 1 M Na_2HPO_4, pH 8. After mixing, urine samples were extracted, evaporated and reconstituted in the same manner as for plasma, using a 1.3-ml volume.

2.7. Method validation

Intraday accuracy and precision for plasma and urine were determined by analyzing replicate

Table 5
Plasma freeze–thaw stability QCs

Cycle	n	Mean calculated concentration[a] (ng/ml)		
		F/T QC 1	F/T QC 50	F/T QC 500
1	2	0.99	48.49	457.9
2	2	1.12	48.63	465.2
3	2	1.07	47.84	494.8
Overall mean		1.06	48.32	472.6
S.D.		0.09	0.73	18.4
CV (%)[b]		8.10	1.52	3.90
% of nominal concentration		105.9	96.6	94.5

[a] Mean calculated concentration of QCs calculated from least squares linear regression: $1/x^2$ weighting of concentration vs. peak area ratio.
[b] Precision represented as % coefficient of variation.

Table 6
Urine freeze–thaw stability QCs

Cycle	*n*	Mean calculated concentration[a] (ng/ml)		
		F/T QC 5	F/T QC 25	F/T QC 250
1	2	4.74	23.48	246.0
2	2	4.89	26.08	268.2
3	2	4.55	22.33	222.4
Overall mean		4.73	23.96	245.5
S.D.		0.17	1.92	21.0
CV (%)[b]		3.53	8.00	8.56
% of nominal concentration		94.6	95.9	98.2

[a] Mean calculated concentration of QCs calculated from least squares linear regression: $1/x$ weighting of concentration vs. peak area ratio.
[b] Precision represented as % coefficient of variation.

calibration curves in each matrix. The calibration curve, composed of all standard replicates, was calculated from weighted ($1/x^2$ for plasma, $1/x$ for urine) least-squares, linear regression analysis of peak area ratios of drug **I** to **IS** versus nominal concentrations. Individual back-calculated concentrations of standard replicates were used to determine mean intraday accuracy at each concentration. Intraday precision was determined by calculating the coefficient of variation (CV) of peak area ratios for replicates at each standard concentration. Initial intraday accuracy and precision were also determined for replicate plasma and urine QC samples, using calculated concentrations.

To evaluate stability on repeat analysis of samples, freeze–thaw stability was determined for three concentrations of **I** in both plasma and urine. QCs were assayed in duplicate before and after each freeze–thaw cycle (samples frozen at −20 °C).

Interday assay variability was determined using plasma and urine QCs. Plasma QCs at low, medium and high concentrations were assayed in duplicate with a standard curve and clinical samples on each day of analysis. The same procedure was followed with urine QCs at low and high concentrations. Acceptance of sample concentration data was based on the validity of QC results.

Assay selectivity was evaluated using five different lots of human control plasma and nine different predose urine samples from subjects enrolled in a Phase I clinical study. Matrix blanks, with and without the addition of known concentrations of **I** and 10 ng/ml of **IS**, were extracted and analyzed ($n = 2$ for control plasma, $n = 1$ for predose urine samples) to assess the presence or extent of interferences.

2.8. Extraction recovery

In determining solid-phase extraction efficiency, plasma or urine blanks were extracted, then reconstituted with appropriate working standard of **I** and working **IS** solution to yield reference calibration curves. Linear regression analysis using peak area (external standard quantitation) was performed to calculate the concentration of **I** recovered from extracted plasma or urine standard replicates versus the reference standard curve. The recovery of **IS** extracted from plasma or urine standards was determined by direct comparison of mean extracted **IS** peak area with that of **IS** in the reconstituted calibration curve.

2.9. Clinical study

Plasma samples from a Phase I, rising dose, study evaluating the safety, tolerability and max-

imum tolerated dose of infused **I** in cancer patients with recurrent or refractory solid malignancies were analyzed using the HPLC-MS/MS described.

3. Results and discussion

3.1. Assay development and collision induced dissociation (CID) fragmentation pathways of drug I

Q1, production and MRM spectra for **I** and **IS** were obtained by infusion. Product-ion spectra and fragmentation patterns are shown in Fig. 1. $(M+H)^+$ ions were *m/z* 406 for drug and *m/z* 448 for internal standard. Precursor/product ions chosen for MRM for **I** and **IS** were *m/z* 406 → 195 and *m/z* 448 → 195, respectively, based on the most intense product ion. Collision energy for **I** was first optimized for sensitivity and resolution using direct infusion of the drug in MeOH.

Fig. 1 shows the product spectra for **I** from the Sciex API 3000, showing the most intense fragment ion *m/z* 195. Based on the structure of the parent molecules, this can be figured as resulting from the cleavage between imidazole and methylene-piperazinone followed by loss of a carbon monoxide. Thus, 195 results from secondary ionization of the parent ion for **I** or **IS**. For the **IS**, the cleavage between methyl, propyl imidazole and piperazinone results in product ion *m/z* 238. Product ion *m/z* 326 most likely resulted from inclusion of chlorophenyl piperazinone moiety and loss of methylene imidazole, $C_4H_4N_2$, (80 amu) from protonated **I**; this same mechanism can explain loss of $C_7H_7N_2$ resulting in product ion *m/z* 123 for the **IS**, shown in Fig. 1. Accordingly for **I**, the product ion *m/z* 298 may be interpreted as the loss of a CO from ion *m/z* 326. For **I**, a product ion at *m/z* 116 is from cleavage of the cyanobenzyl group.

Standards of **I** were chromatographed on a 100 × 4.6 mm C8 analytical HPLC column using trial mobile phases containing 50% ACN, 4% MeOH and 46% of one of the following: (a) 0.05% TFA or (b) 2.5 mM ammonium formate pH 3.0. Optimal for HPLC-MS/MS of **I** and **IS**, with respect to peak shape and signal-to-noise ratio (S/N), was a mobile phase consisting of ACN: MeOH: TFA 0.05% (50, 4, 46 v/v/v). A mobile phase containing 2.5 mM ammonium formate, pH 3.0, in place of 0.05% TFA, resulted in longer retention times for **I** and **IS** (1 min longer for each), broader peaks (>1-min peak width at the base) and increased baseline noise perhaps in part from ionization of the mobile phase (>300 cps background). Signal to noise was the best with 0.05% TFA, compared to 2.5 mM ammonium formate. Consistent peak areas and retention times for solutions containing **I** and **IS** which were injected repeatedly overnight demonstrated stability using 0.05% TFA in the mobile phase.

An extraction procedure using solid-phase-extraction (SPE) was investigated using C8 (EC), C2 and CN (EC) cartridges. The use of a CN cartridge was optimal for sensitivity of the method, and accuracy and reproducibility of peak integration for both I and IS. Although the recovery of both analytes from C8 and C2 cartridges was similar to that from CN cartridges (using peak area for comparison); peak tailing and noise were more prominent for I and IS eluted from C2 and C8 cartridges resulting in reduced peak heights (71–78%) compared to CN cartridges. A possible explanation is that CN cartridges were more selective than C8 or C2 cartridges in retaining I and excluding other organic components in the plasma. The CN cartridges contain cyano propyl groups bonded to endcapped silica. I contains two benzene rings, a cyclic amide, aromatic nitrogens and a cyano group; thus retention on the cartridge is most likely the result of polar (dipole–dipole) and hydrophobic interactions. Also both the CN packing and I have some similarities in chemical structure, both contain cyano groups bonded to carbons, 'like dissolves like'. A high percentage of methanol is required for elution. However, when I was eluted with 90% MeOH in water, the eluate contained particulates, most likely denatured protein, that caused analytical column backpressure to increase over time. This problem was remedied by eluting I with 80% MeOH in water and by using a 2-μm stainless steel pre-filter preceding the analytical column. The 2-μm frit was changed each day before plasma sample analysis, and was not necessary for urine assays.

In addition to selection of cartridge and suitable elution conditions, the following reconstitution solvents were investigated: (a) 1:1 ACN/ammonium formate 2.5 mM, (b) 1:1 ACN/water and (c) 1:1 ACN/0.05% TFA. The optimal reconstitution solvent with respect to peak shape (based on peak width and height), linearity and sensitivity was 1:1 ACN/H_2O. Chomatographic peaks for **I** increased in height by more than 20% when 1:1 ACN/water was used as a reconstitution solvent instead of 1:1 ACN/0.05% TFA or 1:1 ACN/ammonium formate 2.5 mM. The result of sharper peaks from injection of 1:1 ACN/water is most likely a result of superior focusing of the sample on the analytical column in this mixture as opposed to mixtures that contain acids or buffers.

When **I** was added to urine from different sources and then extracted, there was considerable lot-to-lot variability in recovery. Coefficients of variation (CVs) for standard replicates in different control urines ranged from 11 to 19% based on peak area ratio. Recovery appeared to decrease with decreasing urine pH. In the most acidic control urine investigated, only 16% of **I** was recovered. When the same urines were adjusted to pH 8.0 with phosphate (final concentration 0.1 M) there was good agreement among peak area ratios, with CVs $< 6\%$. This lot-to-lot variability was not observed with heparinzed plasma which has a pH of about 8. Thus, to achieve acceptable recovery and reproducibility of **I** from different lots of urine, it was necessary to adjust the urine to pH 8 or higher (Fig. 2).

3.2. Assay selectivity and sensitivity

The selectivity of the method was assessed by analyzing blanks concurrent with calibration standards. Drug **I** and internal standard **IS** were chromatographically separated from each other and from endogenous components in both plasma and urine. Retention times of **I** and **IS** were 3.0 and 3.7 min, respectively, for the plasma method and 4.4 and 5.3 min, respectively, for the urine method.

Representative ion chromatograms of human control plasma are presented in Fig. 3. Extracts of predose plasma samples from clinical subjects were also free of endogenous interferences (Fig. 4). Additionally, no cross talk was observed between the channels for **I** and **IS** on the mass spec. Five different sources of control plasma spiked with 0.5, 1 and 10 ng/ml **I** (1.2, 2.5 and 25 nM, respectively) yielded a mean ($n = 15$) calculated concentration of **I** that was 95.6% of nominal, demonstrating selectivity of the plasma assay. Fig. 5 illustrate the absence of endogenous interferences and cross talk in ion chromatograms of control urine and predose urine samples, respectively. Nine different samples of clinical subject predose urine spiked with 2.5, 5.0 and 250 ng/ml of **I** (6.2 nM, 12.4 nM and 0.62 μM, respectively) yielded a mean ($n = 27$) calculated concentration of **I** that was 106% of nominal, demonstrating selectivity of the assay for **I** in urine. The lower limit of reliable quantitation (LLOQ) was 0.5 ng/ml for the plasma assay and 2.5 ng/ml for urine.

3.3. Linearity, accuracy and precision

The plasma method was initially validated with standards ranging from 0.5 to 100 ng/ml ($n = 6$ replicates). When analysis of clinical samples began, high plasma concentrations of **I** in clinical samples indicated the need to expand the standard curve up to 1000 ng/ml ($n = 5$ replicates). When the assay was re-validated over this extended curve range, the curve remained linear (correlation coefficient of 0.998). Using $1/x^2$ weighting, a typical linear regression equation for **I** in plasma yielded a slope of 0.072 ± 0.010 and a y-intercept of -0.001. Correlation coefficients of ≥ 0.995 were typically obtained, demonstrating linearity over the entire standard range. Intraday accuracy for plasma standards ($n = 5$–6) ranged from 95.4 to 114.4% of nominal concentration, and CVs for precision ranged from 1.8 to 7.8% (Table 1). Plasma QCs were prepared three times over the course of 9 months. Intraday accuracy for each set ($n = 5$) ranged from 90.2 to 104.0% of nominal concentration, and CVs from 0.6 to 6.3%. Intraday QC data for a representative set of QCs is shown in Table 2. Mean accuracy for a representative set of plasma QCs assayed over 3 months was within $\pm 4\%$ of nominal at all concentrations, and inter-day CVs were $\leq 7.2\%$, also shown in Table 2.

A typical linear regression equation for **I** in urine, calculated using $1/x$ weighting, yielded a slope and y-intercept similar to plasma. Typical correlation coefficients of ≥ 0.997 for urine calibration curves demonstrated linearity over the entire standard range. For urine standard replicates ($n = 5$), intraday accuracy ranged from 90.4 to 104.5% of nominal concentration, and CVs for intraday precision ranged from 0.6 to 5.8% (Table 3). Intraday accuracy for both low and high urine QC replicates ($n = 5$) was 94.7% of nominal and CVs were 2.6 and 1.2%, respectively (Table 4). Urine QCs were assayed over the course of 1 month; mean accuracy for urine QCs was 91.5–100.3%, and interday precision was $< 7.1\%$ (Table 4).

3.4. Stability

The stability of **I** was demonstrated in plasma and urine QCs with three freeze–thaw cycles over 3 months. Mean interday assays of plasma freeze–thaw QCs (Table 5) ranged from 94.5 to 105.9% of nominal, with CVs $\leq 8.1\%$. For urine freeze–thaw QCs, mean interday assays ranged from 94.6 to 98.2% of nominal, with CVs $\leq 8.6\%$ (Table 6).

3.5. Recovery

The mean extraction recovery of **I** from replicate plasma standards ($n = 40$ total) was $88.6 \pm 6.6\%$, over the concentration range of 0.5–100 ng/ml. For urine standard replicates ($n = 48$ total) over the concentration range of 2.5–500 ng/ml, the mean extraction recovery for **I** was $90.8 \pm 11.3\%$. In both matrices, the recovery of the **IS** was similar to that of the drug **I**.

3.6. Analyses of clinical samples

The HPLC-MS/MS assay was used to analyze samples from a Phase I clinical study in human cancer patients receiving continuous infusions of **I** at rising doses for 1 week. The plasma concentration time data derived from this study demonstrates the utility of the assay over large concentration ranges [6]. Analysis of urines from eight subjects showed $< 1\%$ or the dose was excreted into the urine.

4. Conclusion

Sensitive and selective methods were developed for the determination of **I** in human plasma at concentrations ranging from 0.5 to 1000 ng/ml, and in human urine from 2.5 to 500 ng/ml. These methods were successfully applied to the analysis of samples from cancer patients. This method may have utility in the assay of other imidazole-based FTIs such as R115777 (Janssen), BMS-214662 (Bristol-Myers Squibb) and additional FTIs (Merck) from plasma and urine. In order to determine the origin of the major product ions m/z 196 and m/z 326, accurate mass determinations were needed. These determinations revealed that the product ion m/z 195 was the result of secondary ionization of **I** and **IS**.

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