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High-throughput sample preparation procedures for the quantitation of a new bone integrin $\alpha_v\beta_3$ antagonist in human plasma and urine using liquid chromatography–tandem mass spectrometry

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Abstract

High throughput LC–MS/MS assays to quantitate a new $\alpha_v\beta_3$ bone integrin antagonist (**I**) in human plasma and urine have been developed using instruments programmed to automate sample preparation procedures. Packard liquid handling system-MultiPROBE® II EX was programmed for preparing calibration standards in control plasma and urine, acidifying all standards, quality control (QC), and clinical samples with necessary dilutions, and adding the internal standard to the acidified samples. TOMTEC Quadra 96™ was programmed to perform the solid phase extraction (SPE) process on a 3 M 96-well mixed phase cation standard density (MPC-SD) plate to isolate the analytes from the sample matrix. The extract collected from both types of matrices was directly injected into reversed-phase LC–MS/MS system with a Turbo Ion Spray (TIS) interface in the positive ionization mode. The plasma and urine assays have the calibration range of 0.5–1500 and 2–6000 ng/mL, respectively. Validation of the automated and the manual plasma assays showed that application of MultiPROBE II to sample preparation gave comparable accuracy and precision. Overall, the automated approaches with minimum manual intervention enhanced the throughput of sample preparation.
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Keyword: Integrin

1. Introduction

Drug **I** (Fig. 1) is a novel orally active $\alpha_v\beta_3$ bone integrin antagonist developed for the treatment and prevention of osteoporosis by inhibiting bone resorption [1,2]. Clinical pharmacokinetic studies for drug **I** required plasma and urine assays with high efficiency to keep pace with the high-volume of clinical studies. In response to this need, high throughput bioanalytical approaches were the focus of the assay development for the quantitation of drug **I** using LC–MS/MS.

Traditional manual sample preparation approaches handling individual samples sequentially have been time-consuming and labor-intensive and thus rate limiting to deliver prepared samples for LC–MS/MS analysis. Automated methodologies utilizing robotic liquid-handling technologies have been increasingly used in the pharmaceutical industry [3–5]. To enhance sample throughput for analysis, automated technologies have been applied to sample preparation and extraction in a 96-well plate format, and allowed for increased preparation capacity with reduced processing time. The challenge of the automated approaches for drug **I** was to design and optimize each instrumental program for high throughput sample preparation without sacrificing the assay quality in terms of accuracy and precision compared to manual processes. The focus of this work was to demonstrate the semi-automated sample preparation using Packard liquid handling system—MultiPROBE® II EX and TOMTEC Quadra 96™. Drug **I** was used as an example for the application of high throughput procedures. The automated procedures described herein are applicable to any compound.

MultiPROBE® II was programmed to prepare all samples with a destination plate in 96-well format. All liquid transfer steps including preparation of calibration standards and quality controls (QC), transfer of clinical samples with necessary dilutions, and addition of internal standard were performed automatically on the MultiPROBE® II.

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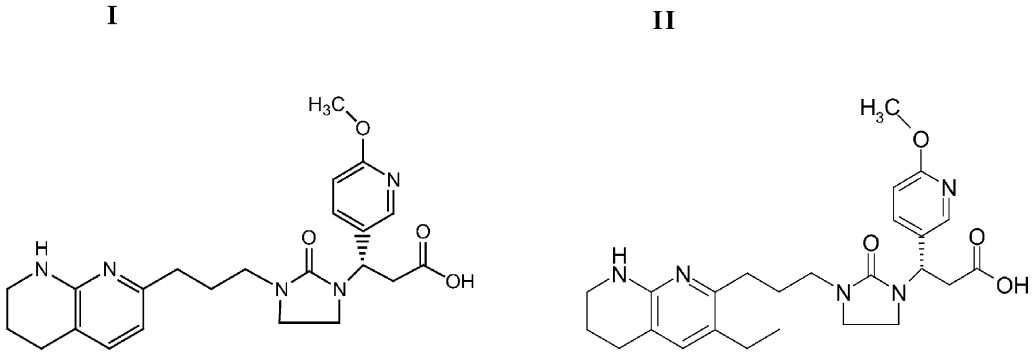

Fig. 1. Chemical structures of the drug **I** and the IS **II**.

The TOMTEC Quadra 96™ was programmed to perform the solid phase extraction (SPE) process on a 3 M 96-well mixed phase cation standard density (MPC-SD) plate to isolate the analytes from 0.1 mL of sample matrix, both plasma and urine, respectively. The resulting extracts collected from both types of matrices were directly injected onto reversed-phase LC system with a LEAP 96-well plate autosampler and analyzed using a mass spectrometer interfaced to a Turbo Ion Spray (TIS) inlet with multiple reaction monitoring (MRM) in the positive ionization mode. The calibration ranges of plasma and urine assays were 0.5–1500 and 2–6000 ng/mL, respectively. Manual and automated sample preparation for the plasma assay procedures have been compared regarding the quality of the assay via intra-day validation.

2. Experimental

2.1. Material

Compound **I** (purity 99.9%) and the internal standard (IS) **II** (purity 98.0%, an analogue of **I**, Fig. 1) were obtained from Merck Research Laboratories (West Point, PA, USA) and used as received. Optima methanol, ACS reagents sodium dibasic phosphate, ammonium hydroxide, HPLC grade *o*-phosphoric acid 85%, laboratory grade formic acid (90%), and ammonium formate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Drug-free heparinized (sodium heparin) human plasma was purchased from Biological Specialty (West Point, PA, USA). Water was purified by Milli-Q from Millipore (Bedford, MA, USA). All chemicals were used as received. A 3 M (St. Paul, MN, USA) Empore high performance mixed phase cation standard density 96-well extraction disk plate was purchased from Phenomenex (Torrance, CA, USA).

2.2. Standard solutions and QC samples preparation

Primary stock solutions of **I** and **II** were prepared at 100 μg/mL and 25 μg/mL in 50/50 methanol/water (v/v), respectively. The stock solution of **I** was further diluted with 50/50 methanol/water (v/v) to obtain a series of working standard solutions from 1 to 3000 ng/mL (1, 2, 10, 20, 100, 200, 1000, 2000, 3000 ng/mL) for plasma assay, and from 4 to 12000 ng/mL (4, 10, 20, 200, 2000, 4000, 8000, 10,000, 12,000 ng/mL) for urine assay, respectively. The working IS solutions prepared by diluting the stock of **II** were 10 and 50 ng/mL in 50/50 methanol/water (v/v) for plasma and urine assays, respectively. All stock and working standard solutions were prepared manually in 10 mL glass tubes with screw caps and stored at −20 °C.

A primary QC stock solution of **I** (100 μg/mL) was prepared from a new weighing in 50/50 methanol/water (v/v), and diluted with 50/50 methanol/water (v/v) to obtain the secondary stock solution (1.0 μg/mL). Three levels of pooled plasma and urine QC samples were prepared by adding appropriate volume of the primary and secondary stock solutions, 1.0, 100, and 900 ng/mL for plasma assay, and 10, 500, and 5000 ng/mL for urine assay, respectively. Aliquots (400 μL) of the QC samples were transferred into polypropylene (pp) centrifuge tubes and stored at −20 °C until analysis.

2.3. Automated 96-well sample preparation on Packard MultiPROBE® II EX

A four-probe Packard (Meriden, CT, USA) liquid handling system MultiPROBE® II EX was programmed for preparing calibration standards in control plasma and urine, acidifying all standards, QCs, and clinical samples with necessary dilutions, and adding the internal standard to the acidified samples in 96-well destination plate. As a routine evaluation regarding accuracy and precision of the liquid transfer procedure, the four probes of the MultiPROBE® II EX were calibrated periodically.

Frozen control plasma and urine, plasma and urine QCs, and clinical samples were thawed at room temperature, mixed by vortex and centrifuged at 2060 × *g* for 10 min. Aliquots (>150 μL) of working standards were transferred manually into pp centrifuge tubes. These working standard pp tubes, QC pp tubes, along with empty pp tubes used

for sample dilution were placed in the designated positions on the test tube rack. All clinical samples contained in pp centrifuge tubes were positioned on the aluminum HS 01 Sample Centrifuge Racks (Horizon Specialty, Inc., PA, USA). This sample rack includes six individual blocks with a total of 96 positions equally spaced, each block containing 16 positions with a 4 × 4 pattern. Once samples were placed on the block, they remained in it through the thaw, vortex and centrifuge process without additional transfers until sample preparation was finished. This approach saved time and effort by minimizing the need for manual transfer of individual sample tubes.

On the Packard MultiPROBE® II EX using a liquid transfer performance file and procedure for each step, 0.4 M sodium phosphate buffer (pH 2) was transferred into each well to acidify each sample: 150 μL of buffer for plasma; 300 μL for urine, which was optimized for assay sensitivity and dynamic range. Aliquots of 100 μL control plasma and urine were transferred into the positions of a 96-well destination plate designated for the preparation of the calibration standards. Then, 50 μL aliquots of the corresponding working standards were spiked into the wells assigned to obtain calibration standards for plasma and urine assays over the range of 0.5–1500 and 2–6000 ng/mL, respectively. Into assigned test tubes, clinical sample dilutions (10-, 100- and/or 500-fold) were performed as needed by transferring diluent (control matrix) and an appropriate aliquot of the sample that required dilution. Next, 100 μL aliquots of the QCs and all clinical samples, undiluted and diluted, were transferred into the 96-well destination plate. In the final step, a 50 μL aliquot of working IS was added to each sample in the destination plate. Plates were capped and mixed by vortex for homogenicity prior to SPE process.

2.4. Manual 96-well sample preparation

Plasma sample preparations as described in the section "Automated 96-well Sample Preparation on Packard MultiPROBE® II EX" were performed manually over the same concentration range using the same type of 96-well plate. An aliquot of control plasma and the phosphate buffer were transferred into a 96-well plate using a repeating pipette (Eppendorf Repeater Plus) with a 5 mL combitip. All aliquots of the working standards, QC samples and working IS were transferred using an electronic pipette (Eppendorf Pipette 100 μL) into designated positions in the 96-well plate, step-by-step accordingly. Plates were capped and mixed by vortex for homogenicity prior to SPE process.

2.5. Automated 96-well SPE process on TOMTEC Quadra 96

TOMTEC (Hamden, CT, USA) Quadra 96–96-well Solvent Delivery System Model 320 was programmed to perform the SPE process using 3 M 96-well mixed phase cation plates for both plasma and urine assays, respectively. The MPC plate was conditioned with 500 μL of methanol, water and 0.4 M phosphate buffer pH 2, sequentially. The acidified sample in the destination plate was loaded onto the MPC plate, 350 μL of plasma and 100 μL of urine, respectively. The MPC plate was washed with 350 μL water, 2% formic acid and methanol, sequentially. Then, the analytes were eluted with 250 μL of methanol/water/ammonium hydroxide 70/28/2 (v/v/v). The eluent was mixed with 100 μL of 2% formic acid added to the collection plate prior to the eluting step. The acid solution balanced the components of the final extract regarding pH and percentage methanol to match with mobile phase content, eliminating the solvent drying and reconstitution steps. The final extract collected from both types of matrices was mixed by vortex, and then directly injected using a LEAP 96-well plate autosampler interfaced with the LC–MS/MS system.

2.6. Chromatographic conditions

HPLC was performed using a Perkin-Elmer Series 200 LC micropump (Thornhill, Ontario, Canada) equipped with a LEAP HTC PAL autosampler (Carrboro, NC, USA) refrigerated at 5 °C during analysis. Chromatography was performed on a BDS Hypersil C18 column, 50 mm × 2 mm, 3 μm (Keystone Scientific, Bellefonte, PA, USA) at room temperature with an injection volume of 5 μL. The mobile phase, consisting of 60% methanol and 40% 5 mM ammonium formate buffer, pH 3 (v/v), was filtered and degassed through a Rainin Nylon-66 0.2 micron filter (Rainin Instrument Co. Inc., Woburn, MA, USA), and delivered at a flow rate of 0.16 mL/min.

2.7. Mass spectrometry conditions

The LC system was interfaced via a Turbo Ion Spray inlet to a PE Sciex (Toronto, Canada) API 3000 triple quadrupole mass spectrometer with the operating software Sample Control (Version 1.4). The mass spectrometry analyses for **I** and **II** were conducted in positive ionization mode. Precursor ions as protonated molecular ions $[M + H]^+$ for **I** and **II** were obtained by infusing neat solution of each analyte using Q1 full scan from *m*/*z* 100 to 500 with the collision gas off. The predominant precursor ions for **I** and **II** were observed at *m*/*z* 440 and 468, respectively. Then, product ion scan was performed for each of the precursor ions under the collision-induced dissociation to obtain fragmentation for each analyte. Representative product scan spectra for the protonated molecule $[M + H]^+$ of **I** and **II** are shown in Fig. 2. The major product ions selected for the analysis were *m*/*z* 261 for **I** and *m*/*z* 289 for **II**, respectively. The state file parameters and instrument settings were optimized to maximize the response for **I** precursor → product ion transition at *m*/*z* 440 → 261. The

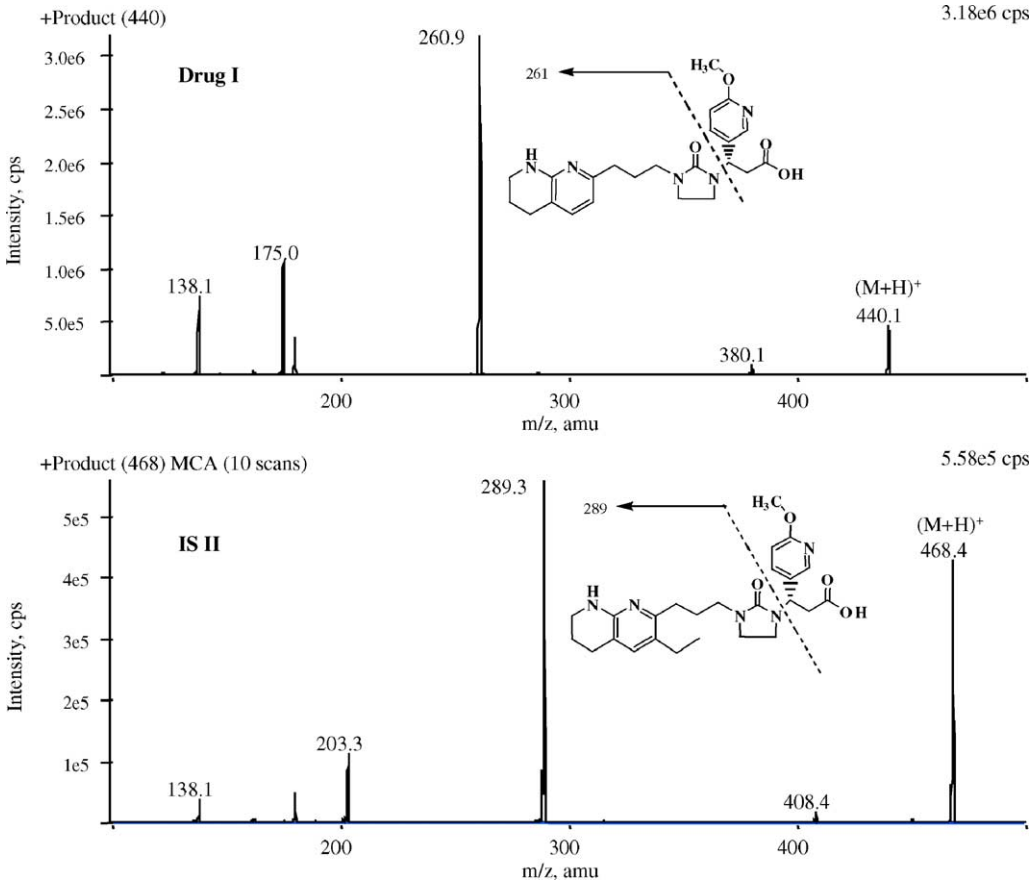

Fig. 2. Representative MRM positive product ion mass spectra for drug **I** and IS **II**.

TIS interface was maintained at 400 °C with the ionspray voltage at +4500 eV, the orifice potential at +55 eV, and the collision cell rod offset at −50 eV. Nebulizer gas (N_2) flow was set at 8000 cc/min. Nitrogen was used as the curtain and collision gas with settings at 10 for both. The analytes were detected by monitoring the precursor → product ion transition using multiple reaction monitoring scan mode, with 300 ms dwell time and 5 ms pause time for each transition. The MRM was performed at *m/z* 440.4 → 261.3 for **I** and *m/z* 468.3 → 289.3 for **II** detecting channel, respectively.

2.8. Analytical data processing

Chromagraphic data were collected and integrated by MacQuan Version 1.6 (Sciex, Toronto, Canada) data analysis program. Peak area ratios of the analyte **I** to IS **II** were utilized for construction of calibration curves. Unknown sample concentrations for both plasma and urine clinical samples were calculated from the equation $y = mx + b$ using peak area ratio versus nominal concentrations and weighted $1/y^2$ [least-squares linear regression analysis of the calibration curve, weighted $\alpha = 1/(\text{peak area ratio})^2$].

3. Results and discussion

3.1. Method development

3.1.1. pH condition

As shown in Fig. 1, **I** contains both acidic and basic functional groups, carboxylic acid (pK_a 4–5) and the amine and aromatic nitrogens of 1,2,3,4-tetrahydro-1,8-naphthyridine (pK_a 7–8) [9]. At intermediate pH (4 < pH < 8), both the carboxyl and amine groups are ionized, giving the zwitterion form of **I**; in this form, **I** is maximally ionized (even though the net charge is zero) and hydrophilic, and has minimum retention on reversed-phase chromatography. Therefore, acidic conditions (pH 2–4) were selected to maintain the analyte in a cation form for SPE processing and reversed-phase chromatography.

3.1.2. Sample preparation program

Packard MultiPROBE® II EX played a very efficient and critical role in the automation process. The performance files used for each transfer step needed to be optimized, regarding aspiration and dispense procedures based on the type of sample matrix and the amount of volume involved. For a routine evaluation of system performance regarding

accuracy and precision of the liquid transfer procedure, four probes of MultiPROBE® II EX were calibrated periodically with Milli-Q water using Mettler SAG285/L Balance placed on the workstation with Gravimetric Performance Evaluation Option provided by Packard. As a requirement, liquid transfer over all critical volumes used in each assay was calibrated with five replicates for each volume. Accuracy and precision for each calibrated volume were within a range of 97–103% and less than 2%, respectively, the requirements of our standard operation procedure (SOP).

Compared to the manual calibration for the MultiPROBE® II, the automated program provided greater efficiency by saving time and labor, and improved accuracy of the measurements by reducing water evaporation with less weighing time, especially for low volumes. The manual calibration process for the MultiPROBE® II involved weighing the aliquots of water transferred by each probe manually one by one on an analytical balance. The lowest volume calibrated using the automated calibration program with disposable micro conductive tips was 10 μL, which has not been successful using the manual process based on the SOP criteria at current laboratory conditions.

Liquid transfer performance files and procedures used for sample preparation on the Packard MultiPROBE® II EX were optimized for each step in the assays in terms of efficiency and accuracy. Except for transferring buffer and matrix using fix tip with adequate washing between each transfer with water, disposable tips were used to transfer working standards, QCs and clinical samples, in order to eliminate possible carryover or contamination problems that might occur with the fix tip. Therefore, there was no need for additional washing steps. All buffers, matrices, and IS were contained in the different troughs placed on the workstation.

The performance of the MultiPROBE® II EX program for each assay was evaluated with regards to specificity, precision and accuracy via the intra-day validation (Tables 1 and 2), over the calibration ranges of 0.5–1500 and 2–6000 ng/mL for plasma and urine assays, respectively. Evaluation of the sample dilution process in the plasma and urine assays was performed by diluting H-QC samples of each assay with control matrix. Representative evaluation results of sample dilutions used in both assays at dilution factors 10, 100, and 500 are shown in Table 3.

3.1.3. Extraction process optimization

The mixed phase cation SPE 96-well plates were employed in the SPE process for sample clean up prior to LC/MS/MS analysis in both plasma and urine assays. MPC sorbent is a silica-based particle that has been bonded with both a reversed-phase group (octyl, C8, non-polar phase) and a strong cation exchange group (benzene sulfonic acid). This mixed phase provides two primary modes of attraction, reversed-phase and cation exchange interactions, and allows for a more efficient and selective extraction compared to traditional reversed-phase techniques. pH 2–4 was used in SPE process to ensure that amine group is fully protonated, which favors the cation exchange interaction; also, to make the carboxyl group remain neutral, which favors the reversed-phase interaction. The analytes were simultaneously extracted from the complex mixture by either reversed-phase and/or cation exchange interactions.

Among the different eluting solvent combinations investigated, acetonitrile/isopropanol/ammonium hydroxide,

Table 1
Intra-day accuracy and precision of drug **I** in human plasma using automated sample preparation ($n = 5$)

Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL)	Mean accuracy (%)[a]	Precision (% CV)[b]
Standard[c]			
0.5	0.508	102	4.43
1	1.00	99.7	9.49
5	4.87	97.3	3.13
10	10.1	101	3.79
50	52.1	104	3.65
100	104	104	5.22
500	500	100	3.40
1000	1000	100	0.654
1500	1430	95.3	1.96
QC			
1	0.989	98.9	5.24
100	103	103	0.692
900	904	100	3.28

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100 (%).
[b] Coefficient of variation based on peak area ratios.
[c] Based on weighted $1/y^2$ linear regression analysis, $y = 0.182x + 0.009$, $r^2 = 1.000$.

Table 2
Intra-day accuracy and precision of drug **I** in human urine using automated sample preparation ($n = 5$)

Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL)	Mean accuracy (%)[a]	Precision (% CV)[b]
Standard[c]			
2	2.02	101	6.70
5	5.07	101	6.39
10	9.75	97.5	4.76
100	103	103	2.44
1000	1030	103	3.09
2000	2000	100	2.20
4000	3930	98.3	2.89
5000	5020	100	4.88
6000	5910	98.5	2.06
QC			
10	9.73	97.3	7.16
500	498	99.6	4.40
5000	5060	101	2.78

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100 (%).
[b] Coefficient of variation based on peak area ratios.
[c] Based on weighted $1/y^2$ linear regression analysis, $y = 0.042x + 0.011$, $r^2 = 0.999$.

Table 3
Representative sample dilution evaluation for drug **I** by diluting HQC with control plasma and urine matrices

Control matrix	Nominal HQC conc. (ng/mL)	Dilution factor	Mean calculated conc. (ng/mL)[a]	Accuracy (%)[b]	Precision (% CV)[c]
Plasma	900	10	943	105	3.75
		100	917	102	3.06
Urine	5000	10	5220	104	5.76
		100	5020	100	3.58
		500	4850	97.0	4.80

[a] Mean value of five replicates for each dilution factor.
[b] Expressed as ((mean calculated concentration)/(nominal concentration)) × 100 (%).
[c] Coefficient of variation based on peak area ratios.

ethyl acetate/isopropanol/ammonium hydroxide, ethyl acetate/pentane/ammonium hydroxide and methanol/ammonium hydroxide, the best recovery for both drug and IS was obtained with the eluting solvent of 98/2 (v/v) methanol/ammonium hydroxide. Next, elimination of the evaporation and reconstitution steps was assessed in order to enhance the efficiency of the SPE process by eliminating the time-consuming steps. The eluting solvent of methanol/ammonium hydroxide was tested with different proportions of water, trying to match the mobile phase content without sacrificing analyte recovery and acceptable sensitivity. The optimal solvent of 78/20/2 (v/v/v) methanol/water/ammonium hydroxide (apparent pH 9, 250 μL) was used as the eluting solvent for both plasma and urine assays. An aliquot of formic acid was added to the eluent to balance the methanol content and pH in the final extract. With 100 μL of 2% formic acid (pH 2) added to each well in the collection plate prior to the eluting step, the final extract was a mixture of the eluent and acid, containing about 50% methanol at a pH 3–4, and matched the mobile phase composition.

3.2. Selectivity, linearity, and sensitivity

Replicate calibration standards of **I** prepared from five different lots of human control plasma and urine, respectively, were assayed to assess within-day variability and selectivity for each method. The selectivity of assay was determined by observing no interfering endogenous peaks in the retention time window of the analyte or IS under the LC–MS/MS conditions for each assay. Representative chromatograms for **I** in clinical plasma and urine samples are shown in Figs. 3 and 4, respectively.

Plasma calibration curves were linear using linear regression weighted $1/y^2$ ($y = 0.182x + 0.009$) in the concentration range of 0.5–1500 ng/mL with a lower limit of quantitation (LLOQ) at 0.5 ng/mL (Table 1). Urine calibration curves were linear using linear regression weighted $1/y^2$ ($y = 0.042x + 0.011$) in the concentration range of 2–6000 ng/mL with a LLOQ at 2 ng/mL (Table 2). Mean correlation coefficients (r^2) from intra-day analysis over five calibration curves were 1.000 and 0.999 for plasma and urine, respectively.

3.3. Intra-day variability

The results of intra-day validation of standards and QC samples using high throughput procedures are listed in Tables 1 and 2, respectively. Accuracy of the plasma assay in the range of 0.5–1500 ng/mL ($n = 5$) was 95.3–104% of nominal, while the precision varied between 0.654 and 9.49% (CV). Accuracy and precision of plasma QC samples at low (L), middle (M), and high (H) concentration ($n = 5$) were 98.9–103% and 0.692–5.24% (CV), respectively. As a comparison, accuracy and precision of plasma calibration standards and QC samples using manual sample preparation over the same range ($n = 5$) are indicated in Table 4. The validation data for manual and automated sample preparation are very similar in terms of accuracy and precision for standards and QC samples, and slopes and correlation coefficients of the linear calibration curves (Tables 1 and 4). For the urine assay over the calibration range of 2–6000 ng/mL ($n = 5$), accuracy and precision varied between 97.5 and

Table 4
Intra-day accuracy and precision of drug **I** in human plasma using manual sample preparation ($n = 5$)

Nominal conc. (ng/ml)	Mean calculated conc. (ng/mL)	Mean accuracy (%)[a]	Precision (% CV)[b]
Standard[c]			
0.5	0.50	99.6	9.48
1	1.05	105	10.0
5	4.99	99.8	3.54
10	10.3	103	5.64
50	51.8	104	5.36
100	103	103	1.55
500	498	99.6	5.18
1000	977	97.7	2.40
1500	1430	95.4	3.50
QC			
1	1.11	111	7.15
100	94.7	94.7	3.43
900	808	89.8	3.58

[a] Expressed as ((mean calculated concentration)/(nominal concentration)) × 100 (%).
[b] Coefficient of variation based on peak area ratios.
[c] Based on weighted $1/y^2$ linear regression analysis, $y = 0.184x + 0.003$, $r^2 = 0998$.

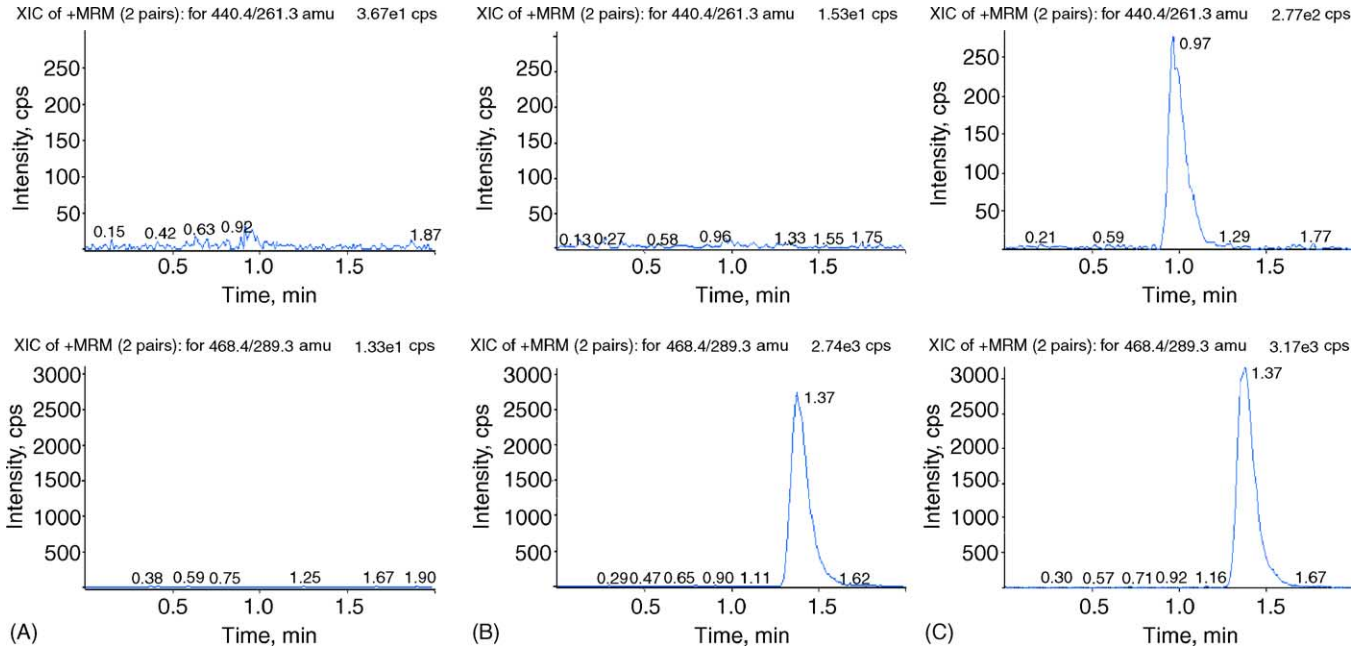

Fig. 3. Representative chromatograms* of **I** in human plasma; (A) plasma double blank; (B) plasma single blank; (C) plasma standard LLOQ 0.5 ng/mL. (*) Each upper chromatogram represents drug **I** channel (*m/z* 440.4 → 261.3), and each lower chromatogram represents **II** (IS) channel (*m/z* 468.4 → 289.3).

103.0% of nominal and 2.06–6.70% (CV), respectively. The accuracy and precision of urine QC samples (L, M, and H) ($n = 5$) were from 97.3 to 101% and 2.78 to 7.16% (CV), respectively.

3.4. Inter-day variability

Inter-day variabilities of the plasma and urine assays were evaluated using sets of low, middle, and high QC samples analyzed daily with clinical samples. These plasma and urine QC samples were used to determine the validity of routine analysis runs. The overall inter-day accuracy and precision data in Table 5 for plasma assay represents 16 sets of plasma QC samples over an 8 month period with three analysts involved. The correlation coefficients for all plasma calibration curves were greater than or equal to 0.997 with the slope ranging from 0.155 to 0.227 (CV% of 14.0). The overall inter-day accuracy and precision data in Table 5 for

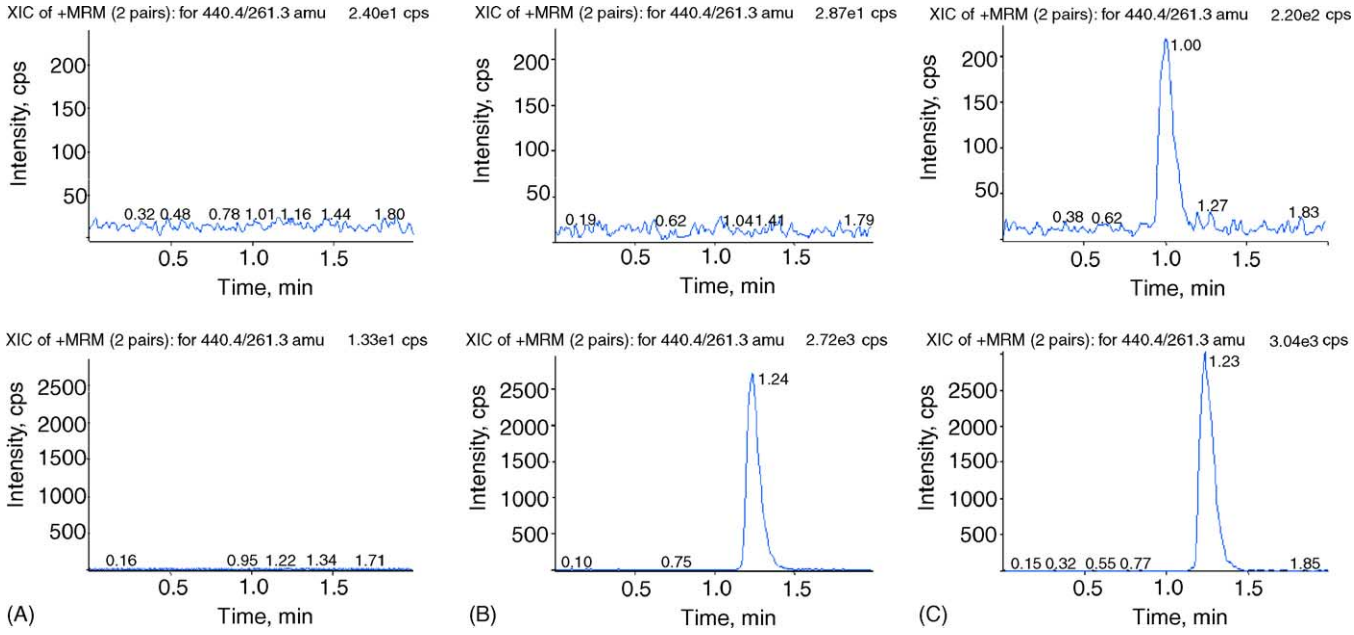

Fig. 4. Representative chromatograms of **I** in human urine; (A) urine double blank; (B) urine single blank; (C) urine standard LLOQ 2 ng/mL. (*) Each upper chromatogram represents drug **I** channel (*m/z* 440.4 → 261.3), and each lower chromatogram represents **II** (IS) channel (*m/z* 468.4 → 289.3).

Table 5
Inter-day QC precision and accuracy for drug **I** in plasma and urine assays

Assay matrix	Nominal conc. (ng/mL)	Mean calculated Conc. (ng/mL)	Mean accuracy (%)[a]	Precision (% CV)
Plasma[b]	1	0.98	98.4	15.9
	100	103	103	12.4
	900	874	97.1	10.4
Urine[c]	10	9.42	94.2	9.01
	500	513	103	7.38
	5000	5102	102	5.75

[a] Based on the nominal of QC samples.
[b] CV (%) representing 16 different sets of QC samples over a 8-month period.
[c] CV (%) representing 23 different sets of QC samples over a 7-month period.

urine assay represents 23 sets of urine QC samples over a 7 month period with two analysts involved. The correlation coefficients for all urine calibration curves were greater than or equal to 0.994 with the slope ranging from 0.032 to 0.044 (CV% of 12.2).

3.5. Assay recoveries, matrix effect, and stability

Assay extraction recoveries and sample matrix effect on ionization in MS/MS for **I** and IS **II** were evaluated for both plasma and urine assays. SPE recoveries were determined by comparing the absolute peak areas of the analytes **I** and **II** spiked before SPE in the blank control matrix and spiked after SPE into the extracts of blank control matrix (post-spiked) at three different concentrations. Recoveries and matrix effects analysis for plasma and urine assays conducted over the calibration ranges of 0.5–1500 and 2–6000 ng/mL, respectively, are shown in Table 6. The overall recoveries of **I** ($n = 5$) over three concentrations and IS **II** ($n = 15$) were about 72.4 and 70.2% in plasma, and 80.0 and 77.4% in urine, respectively. Matrix effects on ionization (suppression or enhancement) of **I** and IS **II** were determined by comparing the absolute peak areas of the post-spiked extract standards to those of the neat prepared in mixture of the eluting solvent and the final balance solution. No significant matrix effects were observed in extracts (Table 6), overall for both **I** and IS **II** in plasma and urine extracts, respectively. In addition, based on the validated intra-day precision and accuracy results of each assay using five different lots of each matrix, the relative matrix effects under current assay conditions were not considered significant with respect to assay performance.

The stability of **I** in stock and working standard solutions, and in plasma and urine QC sample (L, M, and H, $n = 3$) extracts was monitored under bench process conditions at room temperature. Drug **I** in above three matrices was stable (>95% remaining) over 24 h at room temperature. Freeze-thaw stability of plasma and urine QC samples (L, M, and H) was evaluated with three replicates ($n = 3$) at each level over three cycles. Three sets of QC samples at each level were initially quantitated prior to freezing; they were then frozen and thawed up to three cycles prior to quantitative analysis. Freeze-thaw stability studies indicated no effect on **I** content in plasma QC and urine QC samples subjected to three freeze-thaw cycles. The long-term storage stability of drug **I** in plasma and urine at $-20\,^{\circ}C$ was monitored using inter-day QC samples analyzed with clinical samples. Overall, the drug is stable in human plasma and urine at $-20\,^{\circ}C$ storage condition for at least 17 and 8 months, respectively.

4. High throughput efficiency and applications

Generally, the programs for the preparation of three sample plates containing over 270 samples on the Packard

Table 6
Recoveries and matrix effect of drug **I** and IS in plasma and urine assays

Analyte	Sample matrix	Nominal conc. (ng/mL)	% Mean recovery $n = 5$ (SD)[a]	% Mean matrix effect $n = 5$ (SD)[b]
Drug	Plasma	1	72.0 (11.7)	100.3 (5.4)
		100	61.2 (9.5)	107.2 (6.6)
		1000	84.1 (2.0)	96.8 (4.0)
	Urine	5	78.2 (4.2)	99.5 (3.1)
		1000	79.7 (4.1)	89.2 (4.4)
		4000	82.2 (2.7)	89.5 (3.5)
IS	Plasma ($n = 15$)[c]	5	70.2 (6.6)	101 (2.6)
	Urine ($n = 15$)[c]	5	77.4 (3.3)	91.5 (2.0)

[a] Expressed as ((extract mean peak area/spiked mean peak area) × volume factor) × 100 (%).
[b] Expressed as ((spiked mean peak area/neat mean peak area) × volume factor) × 100 (%).
[c] In the presence of drug **I** at three concentration levels.

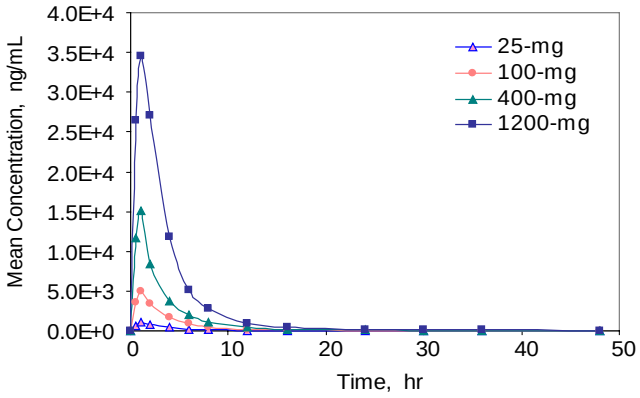

Fig. 5. Mean plasma concentration profiles of drug **I** in six healthy subjects receiving single oral dose of drug **I** at 25–1200 mg doses.

MultiPROBE® II with four-probes can be completed in about 2 h. While the second 96-well sample plate is prepared on the MultiPROBE® II, the first prepared sample plate can be move to the TOMTEC for SPE processing, which takes about 35 min for each plate. The LC–MS/MS analysis of three plates of sample extracts using a 96-well plate autosampler set at a 5 μL injection volume and 2 min run time can be completed overnight. The SPE process provides efficient and effective sample clean up; one analytical column can be used for the analysis of at least 1300 plasma and urine extracts. The automated assays are standardized and easy to be handled by different analysts.

Automated plasma and urine assays have been successfully applied to seven clinical pharmacokinetic studies. Representative mean plasma profiles of the drug **I** from six representative healthy subjects administered a single oral rising dose of drug over the dosing range of 25 to 1200 mg are shown in Fig. 5.

5. Conclusion

Automated LC–MS/MS bioanalytical assays for the high throughput analysis of **I** in human plasma and urine have been developed, validated, and effectively implemented for seven clinical pharmacokinetic studies. Compared to the manual procedure, the high throughput methods provided substantially more efficient bioanalytical support for all clinical studies with adequate precision and accuracy. Efficiency has been improved at least four-fold in instrument utilization with significant time saving over manual procedures. The automated programs on the Packard and TOMTEC instruments for **I** can be easily adapted to other compounds for efficient sample preparation and SPE.

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