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Simultaneous determination of a novel KDR kinase inhibitor and its *N*-oxide metabolite in human plasma using 96-well solid-phase extraction and liquid chromatography/tandem mass spectrometry

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Abstract

To support pharmacokinetic studies, a selective and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the simultaneous determination of a novel KDR kinase inhibitor (**1**) and its active metabolite (**2**) in human plasma. The method is fully automated using a Packard MultiPROBE II system and a TomTec Quadra 96 liquid handling workstation to perform sample preparation and solid-phase extraction (SPE). Following the extraction on a mixed-mode SPE using Oasis MCX 96-well plate, the analytes were separated on a Aquasil C18 column (50 mm × 2.1 mm, i.d., 3 μm) with a mobile phase consisting of acetonitrile/ammonium acetate buffer (5 mM, pH 5.0) (60/40, v/v). The run time for each injection was 4.5 min with the retention times of approximately 2.0 and 2.7 min for **1** and **2** respectively, at a flow rate of 0.25 mL/min. A tandem mass spectrometric detection was conducted using multiple reaction monitoring (MRM) under the positive ion mode with a turbo ion-spray interface. The linear ranges of the calibration curves were 0.05–400 ng/mL for **1** and 0.1–400 ng/mL for **2** on a PE Sciex API 4000 LC–MS/MS system. The lower limits of quantitation (LLOQ) of the assay were 0.05 and 0.1 ng/mL for **1** and **2** respectively, when 0.4 mL of plasma was processed. Intra-day assay precision (using five standard curves prepared by spiking compounds to five lots of plasma) was less than 4.9% for **1** and less than 9.6% for **2** on each concentration. Assay accuracy was found to be 95.1–104.6% of nominal for **1** standards and 93.5–105.6% for **2** standards. QC samples were stable when kept at room temperature for 4 h, at −70 °C for 10 days, and after three freeze–thaw cycles. The extraction recoveries were 80%, 83% and 84% for **1** and **2** and I.S. respectively, and no significant matrix effects were observed. The method was successfully applied to plasma samples from clinical studies after oral administration of compound **1**.

Keywords: KDR kinase inhibitor; *N*-Oxide metabolite; LC–MS/MS; Oasis MCX solid-phase 96-well extraction plate

1. Introduction

The recent development in understanding the biochemical mechanism in the processes of carcinogenesis disclosed that angiogenesis, a process involving the new blood vessel formation needed for tumor growth and metastasis, is one of the important targets for anticancer drug therapy [1,2]. Vascular endothelial growth factor (VEGFs) has been shown to be secreted by tumor cells and microphages, and considered as a key angiogenic factor of tumor-induced angiogenesis [3,4]. The kinase insert domain containing receptor (KDR), also known as VEGRF2 tyrosine kinase (VEGFR-2), is a transmembrane VEGF receptor that possess intrinsic cytoplasmic enzymatic activity, catalyzing the transfer of the gamma-phosphate of ATP to tyrosine residues in protein substrates

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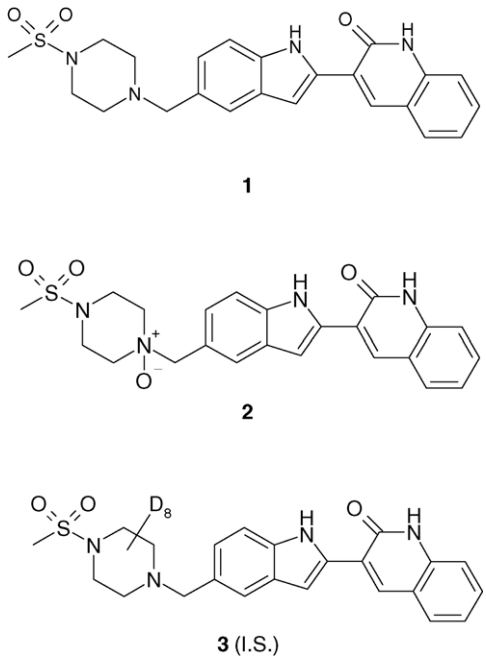

Fig. 1. Chemical structures of compounds **1**–**3**, the internal standard (I.S.).

[5]. It functions as a critical regulator in signal transduction pathways that affect cell proliferation, differentiation, migration and metabolism. Blocking KDR activity limits the VEGF-induced blood vessel formation in most tumors, and thus inhibits growth and metastases of the tumor [6–9]. Since the inhibition of KDR selectively targets the tumor associated vessels, the anticancer therapy through this mechanism is expected to be safe and well tolerated by cancer patients [10].

Compound **1**, 3-[5-(4-methanesulfonyl-piperazin-1-yl-methyl)-1 *H*-indol-2-yl]-1*H*-quinolin-2-one (Fig. 1), is a novel and potent KDR kinase inhibitor, which is under clinical investigation for the treatment of cancer. As part of the drug development process, the pharmacokinetics needs to be determined. Compound **2** is a *N*-oxide metabolite of **1** with comparable potency as its parent compound. Since its concentration appears to be about 10% of parent drug **1** in rat plasma during preclinical pharmacokinetic studies, it will be important to explore the concentration level of **2** in human plasma.

Rapid growth of using 96-well extraction technology in conjunction with LC–MS/MS for the analyses of drugs in biofluids has led to the successful high throughput drug quantification with excellent selectivity and sensitivity [11,12]. Described here is an automated method that utilizes this technology for the simultaneous determination of **1** and its metabolite **2** in human plasma to support the clinical pharmacokinetic studies. The selected extraction technology is solid-phase extraction (SPE) in 96-well format using Waters Oasis MCX product that contains a mixed-mode polymeric sorbent with reversed-phase and cation-exchange functionalities. The conditions for sample preparation, chromatography and mass spectrometer detection were optimized. The assay specificity, extraction recovery and matrix effect were assessed. The method was validated over the ranges of 0.05–400 ng/mL for compound **1** and 0.1–400 ng/mL for compound **2** on a PE Sciex API 4000 LC–MS/MS system, using 0.4 mL plasma sample.

2. Experimental

2.1. Materials and solutions

2.1.1. General materials

Compounds **1** and its metabolite **2** (Fig. 1) were synthesized at the Merck Research Laboratories, Merck & Co. (West Point, PA, USA) [13]. Compound **3** (Fig. 1), containing eight deuterium labels on the piperazine ring of compound **1**, was obtained from Merck Research Laboratories (Rahway, NJ, USA), and used as an internal standard (I.S.) in the assay. HPLC grade acetonitrile and ammonium acetate, optima grade methanol, laboratory grade formic acid (90%) and ACS grade acetic acid and ammonium hydroxide (29.7%) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Human control plasma (sodium heparin as anticoagulant) was purchased from Biological Specialty Co. (Colmar, PA, USA). Water was purified by a Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA). Oasis® MCX (mixed-mode cation exchange) 96-well extraction plate (10 mg/well) was purchased from Waters Corporation (Milford, MA, USA) and used for solid-phase extraction (SPE) of plasma samples.

2.1.2. Solutions for plasma assay

A solution of acetonitrile/0.1% formic acid (50:50, v/v) was used as dissolving solvent, reconstitution solvent after SPE, and washing solvent for both Packard Liquid Handling System and Leap Autosampler. A solution of acetic acid (0.1 M) was prepared to acidify the plasma samples before extraction and wash SPE plate after loading. A solution of methanol/29.7% ammonium hydroxide (95:5, v/v) was prepared as the SPE eluting solvent for compounds **1** and **2** in plasma. The mobile phase for the LC–MS/MS assay was prepared by combining 600 mL acetonitrile, 360 mL Milli-Q water and 40 mL ammonium acetate (50 mM, pH 5.0) which was prepared in advance and its pH was adjusted with acetic acid. The mobile phase was filtered through a 0.2 μm nylon filter prior to use.

2.2. Equipment

For the plasma assay, a Packard MultiPROBE II automated liquid handling system (Meriden, CT, USA) and a TomTec Quadra 96 workstation (Model 320, Hamden, CT, USA) were used to perform sample transferring and automated solid-phase extraction (SPE) process respectively. LC–MS/MS was performed on a LC micro pump (Series 200 from Perkin-Elmer, Ontario, Canada) and 96-Well Plate Autosampler (HTS PAL System from Leap Technology, Carrboro, NC,

USA), coupled to a Sciex API 4000 triple quadrupole mass spectrometer with a Sciex Turbo Ion Spray Interface (Sciex, Toronto, Canada) used in the positive-ion mode. The data were collected and processed through Analyst 1.1 software. During the synthesis of compound **3**, the identities of all intermediates and the final product were established by ^{1}H NMR, using a Varian Unity 400 spectrometer (Palo Alto, CA, USA).

2.3. Chromatographic conditions for plasma assay

The HPLC separation was performed on an Aquasil C18, 50 mm × 2.1 mm column with 3 μm particle size from Keystone Scientific (Bellefonte, PA, USA) with a 10 μL sample injection. The mobile phase was composed of acetonitrile and ammonium acetate buffer (5 mM, pH 5.0) at a ratio of 60/40 (v/v), and its flow rate was 0.25 mL/min. The column temperature was maintained at 35 °C to improve peak efficiency. A mixture of acetonitrile and 0.1% formic acid with a ratio of 50:50 (v/v) was used as the injector washing solution. The autosampler was protected from light exposure, and its temperature was set to 5 °C.

2.4. MS/MS detection and calculation for plasma assay

A PE Sciex API 4000 triple-quadrupole mass spectrometer with a turbo-ionspray interface ionization source operated in a positive ion mode was used to quantitate the analytes. Precursor ions for compounds **1**–**3** (I.S.) were determined from Q1 spectra obtained during the infusion of their neat solutions into the mass spectrometer with the collision gas off. Each of the precursor ions was subjected to collision induced dissociation (CID) to determine the resulting product ions. The ion pairs (precursor ion → product ion), *m/z* 437 → 273 for compound **1**, *m/z* 453 → 273 for **2** and *m/z* 445 → 273 for **3**, were selected for multiple reaction monitoring (MRM). The instrument setting was adjusted to maximize the response for each of the analytes and I.S. respectively during t-shape infusion in the presence of mobile phase. A high voltage of 4.5 kV was applied to the sprayer. The turbo gas temperature was 450 °C and the auxiliary gas flow was 30 L/min. The flow settings of nebulizing gas (nitrogen), collision gas (nitrogen), curtain gas (nitrogen) at the instrument were 55, 4 (CGT = 2.0×10^{15} molecules per cm^2) and 15 L/min, respectively. The optimized declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) were set at 46, 21 and 16 V for compound **1**, 38, 20 and 14 V for **2**, 45, 19 and 19 V for **3**, respectively. The optimized entrance potential (EP) was 5 V for all compounds. The dwell times were 200 ms for compounds **1** and **3** and 300 ms for compound **2**. Both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total acquisition time was 4.5 min. Peak area ratios for calibration curves and quantitation of unknowns were calculated using Analyst software version 1.1. Calibration curve was obtained by weighed ($1/x^2$) least squares linear regression on the peak area ratio of each analyte to the internal standard versus the nominal concentration (x) of each analyte.

2.5. Preparation of calibration standards and QC samples

Internal standard and two separate weighing of each analyte were prepared under yellow light and dissolved in acetonitrile and 0.1% formic acid in the ratio of 50:50 (v/v) to make stock solutions. One set of analyte stock solutions was used to prepare calibration standards, and the other set was used to make QC samples. The concentration of stock solution for each analyte was 40 μg/mL (free base form). Working standards were the mixture of compounds **1** and **2** at the concentration levels of 0.5–4000 ng/mL in acetonitrile/0.1% formic acid (50/50, v/v). They were prepared by serial dilutions from the analyte stock solutions, and stored in amber glass tubes at 4 °C. A 50 ng/mL I.S. working solution was obtained by dilution of 25 μg/mL I.S. stock solution in acetonitrile/0.1% formic acid (50/50, v/v). Plasma calibration standards were prepared daily by adding 40 μL of working standard and 40 μL of 50 ng/mL I.S. into 400 μL of control plasma to provide final concentrations of compounds **1** and **2** in plasma ranging from 0.05 to 400 ng/mL. QC samples were prepared at 0.15, 10 and 200 ng/mL for compound **1** and 0.3, 10 and 200 ng/mL for **2**, aliquoted into 3 mL polypropylene vials and then stored at −70 °C freezer.

2.6. Sample preparation and solid-phase extraction (SPE)

Control plasma, QCs and clinical samples were thawed at room temperature, mixed by vortex, and centrifuged at about 1000 × *g* RCF, 10 °C for 5 min. An aliquot of 400 μL of each sample (or control plasma for standards) was transferred from the vial into a 2 mL 96-well deep-well plate (Matrix Technologies Corp., Hudson, NH, USA) by the Packard MultiPROBE II robotic liquid handler. Between transferring, the MultiPROBE tips were rinsed with water, acetonitrile/0.1% formic acid (50/50, v/v), and then water again. No carryover from sample transfer tips was observed. The following solutions: 40 μL of working standard (for calibration standards) or solvent (for single blank, QC and clinical samples), 40 μL of 50 ng/mL of I.S. working solution and 520 μL of 0.1 M acetic acid were then sequentially added to each well to make total of 1000 mL of acidified sample mix. After vortex, the sample plate was moved to Tomtec Quadra 96 workstation to perform automated SPE using Oasis®MCX mixed-mode extraction plate. Aliquot of 900 μL of the acidified samples was loaded onto the SPE plate under applied vacuum without conditioning step. The sample wells were then washed with 800 μL (transferred 400 μL twice because of the tip volume limitation on Tomtec) of 0.1 M acetic acid, followed by 800 μL of acetonitrile. After drying the SPE plate by centrifugation at about 200 × *g* RCF, 5 °C for 5 min, the analytes were eluted with 0.5 mL of methanol/29.7% ammonium

hydroxide (95/5, v/v) into a new 1 mL deep-well collection plate (MicroLiter Analytical Supplies, Suwanee, GA, USA). The collected eluent was evaporated under a stream of nitrogen at 35 °C on a SPE Dry-96 (John Chromatograph, Lakewood, CO, USA). The residues were reconstituted in 150 μL of acetonitrile/0.1% formic acid (50/50, v/v), and the samples were ready for injection to LC–MS/MS. All procedures were conducted under yellow light due to the light sensitivity of compound **1**.

2.7. Method validation

The selectivity of the assay was confirmed by processing control plasma from six different lots. Intraday precision and accuracy were determined by analyzing five standard curves in five different lots of control plasma. Assay accuracy was calculated from a least-squares regression curve constructed using all five replicate values at each concentration, and the intraday precision (%CV) was calculated from the peak area ratio of parent versus I.S. for each concentration used to construct the standard curve. The final concentrations on the standard curve were 0.05, 0.1, 0.2, 0.5, 2, 10, 20, 100, 300 and 400 ng/mL. QC samples were analyzed after first freezing and thawing, and the measured concentrations were considered as the initial values. The short-term stability was evaluated after the storage of QCs in the freezer for more than 10 days. Freeze–thaw stability was evaluated using QC samples that went through three cycles of freezing and thawing, with at least one-day storage at −70 °C between each thaw. Bench-top stability was tested by comparing the measured concentration of QC samples that has been kept at room temperature under yellow light (experimental condition) for 4 h with their initial data. The stability of processed samples in autosampler at 5 °C was assessed by comparing the results of QC samples analyzed at the end of the run (at least 16 h in autosampler) with those analyzed at the beginning of the run. Reinjection reproducibility was demonstrated by comparing the results of the same five intraday-validation curves analyzed before and after storage at −20 °C for at least 7 days.

2.8. Extraction recovery and matrix effect

SPE extraction efficiency was evaluated at the nominal concentrations of 0.2, 10 and 300 ng/mL ($n = 5$ at each analyte concentration) and the working concentration of 5 ng/mL for I.S. ($n = 15$). Recovery of the extraction was determined by comparing the absolute peak areas of the pre-spiked analyte with those of the post-spiked analyte. The pre-spiked analyte was prepared by the extraction procedure described in Section 2.6. The post-spiked samples were the drug-free control plasma extract prepared by the same manner and then spiked with working stocks containing compounds **1**–**3**.

The matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of the post-spiked standards with those of the neat standards in reconstitution solvent. On the other hand, the matrix effect was also evaluated by performing precision and accuracy determination from five different lots of plasma (see intraday validation).

3. Results and discussion

3.1. Chromatographic and MS/MS conditions

Compound **1**, which contains a methanesulfonyl-piperazine group, was determined to have pK_a values of 7.97 and 3.78 (calculated from the software ACDLabs/pK_a DB version 4.5, Advanced Chemistry Development Inc.). The use of acetonitrile/0.1% formic acid (50/50, v/v) as a mobile phase resulted in either poor retention or tailing peak shape or both on most of the tested C18 columns: Keystone Scientific BDS Hypersil C18, Waters Xterra MS C18, ANSYS Polaris C18-A and Supelco Discovery C18. With a mobile phase of acetonitrile/ammonium acetate buffer (5 mM, pH 5.0) (60/40, v/v), Aquasil C18 column (50 mm × 2.1 mm with 3 μm packing) from Keystone Scientific exhibited the best peak shape and retention characteristics for compounds **1** and **2**. The carryover between each injection was solved by using acetonitrile/0.1% formic acid (50/50, v/v) as a needle washing solvent. The column temperature was set at 35 °C because it sharpened the peak of compound **2** so as to improve its assay precision, especially at the lower limit of quantitation (LLOQ). Compounds **1** and **2** were baseline separated with retention times 2.0 and 2.8 min, respectively.

Precursor ions of compounds **1**–**3** were determined from Q1 scans during the infusion of neat solutions in a positive ionization mode using auto-tuning function in Analyst software. Three precursor ions, *m/z* 437 for **1**, *m/z* 453 for **2** and *m/z* 445 for **3**, were subjected to collision induced dissociation (CID) and tuned simultaneously in order to determine the resulting product ions. Under the optimized fragmentation conditions, one major product ion (*m/z* 273) was present in each of the product ion scans of compounds **1**–**3**, as shown in Fig. 2. Therefore, the mass transition patterns, *m/z* 437 → 273, 453 → 273 and 445 → 273 were selected to monitor compounds **1**–**3** respectively.

Since compounds **1** and **3** (I.S.) gave identical ions in Q3 and co-eluted under chromatographic condition, the "cross-talk" between channels used for monitoring the compound **1** and I.S. was evaluated by analysis of their neat individual solutions at 1000 ng/mL. The responses in the MRM mass transition channels used for quantification were monitored. No "cross-talk" or interference between the compound **1** and internal standard was observed. Since compound **2** was baseline separated from **1** under assay chromatographic conditions prior to its mass spectrometric analysis, the potential interference of in-source fragmentation of **2** was avoided.

Representative extracted ion chromatograms of a control plasma single blank and a plasma standard containing

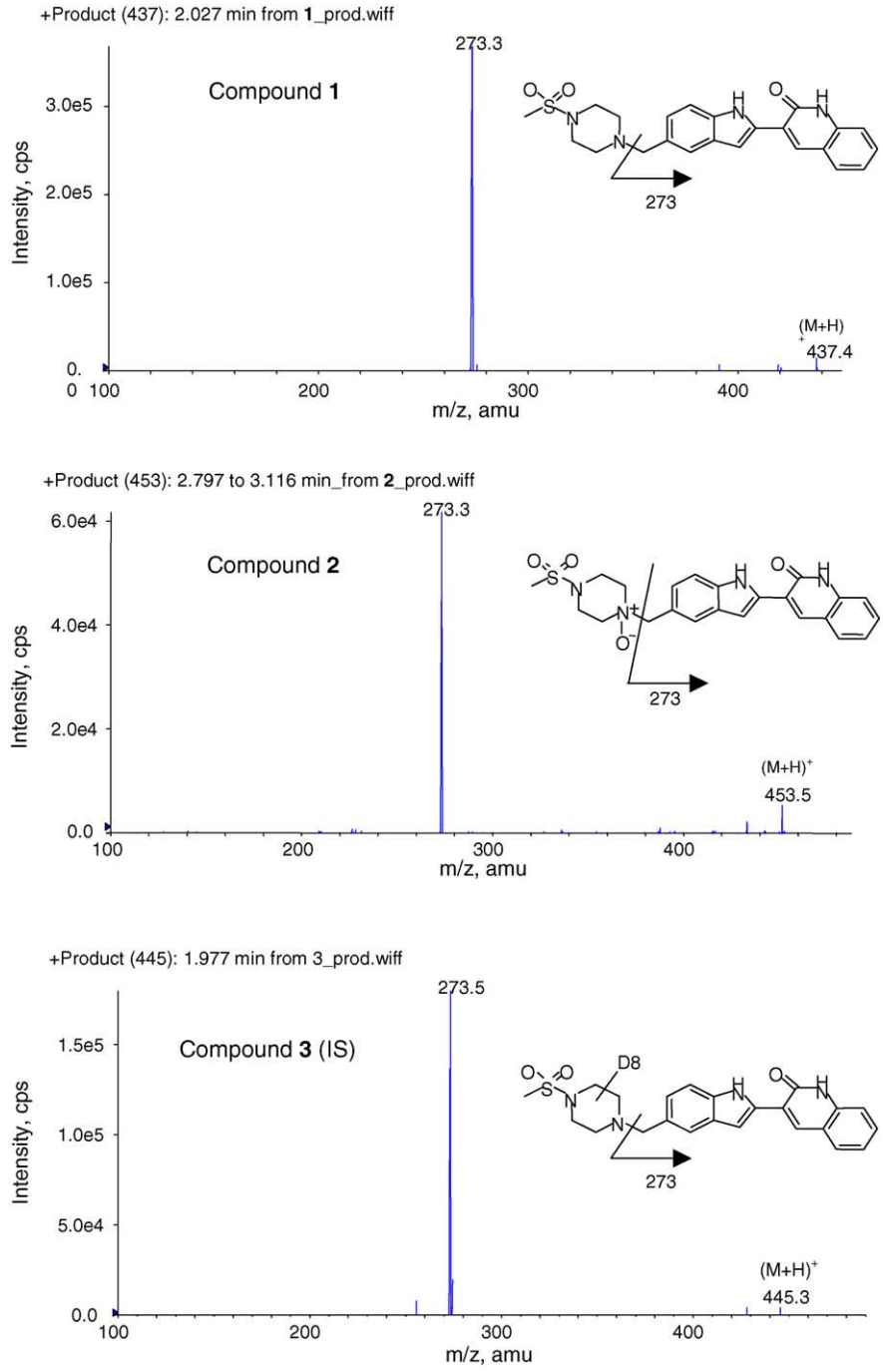

Fig. 2. MS/MS product ion mass spectra of the protonated molecules $(M+H)^+$ of compounds **1** and **2** and I.S. at *m/z* 437, 453 and 445 respectively.

0.05 ng/mL of compound **1** (LLOQ), 0.1 ng/mL of compound **2** (LLOQ) and 5 ng/mL of I.S. are shown in Fig. 3 (Panel A and B).

3.2. Sample preparation, extraction recovery and matrix effect

A selective, high-throughput solid-phase extraction method was developed utilizing automated 96-well plate technology for isolation of **1**–**3** from plasma. Mixed mode (reversed phase and cation-exchange) solid-phase extraction plates were evaluated systematically by using Waters Oasis MCX, 3M Empore™ MPC-SD (purchased from Phenomenex) and ANSYS SPEC MP1 and MP3, all in 96-well plate format. Due to the observation of significant matrix effect (more than 20%) and interference peaks with 3M Empore MPC and ANSYS SPECs, respectively, Oasis MCX was selected as the SPE sorbent of choice. Since Oasis MCX contains strong cation-exchange sulfonic acid groups on the surface of Oasis HLB sorbent, it can

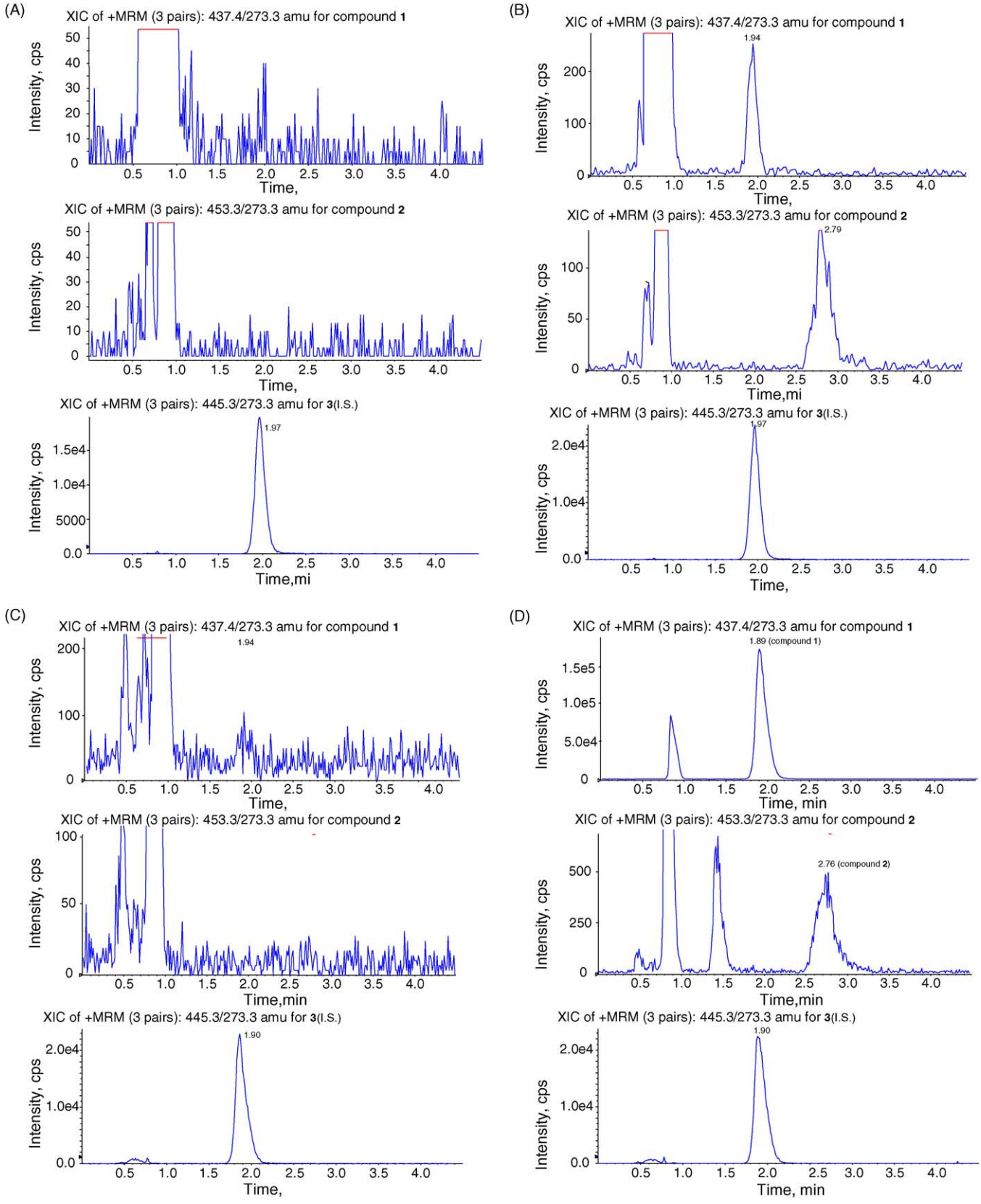

Fig. 3. Representative MRM chromatograms for (A) single blank, control human plasma fortified with 5 ng/mL I.S.; (B) LLOQ, 0.05 ng/mL of compound **1** and 0.1 ng/mL of compound **2** with 5 ng/mL I.S. in human plasma standard; (C) predose plasma sample from subject #001; (D) 4 h post-dose plasma sample from subject #001 following a 12 mg single oral dose of compound **1**. The concentrations found in this sample were 34.5 and 0.319 ng/mL for compounds **1** and **2**, respectively.

allow relatively specific extraction of basic analytes from plasma.

The extraction procedure, including sorbent conditioning, sample loading, washing and eluting steps, was then optimized on a 10 mg Waters Oasis MCX 96-well plate. The SPE recoveries were comparable with or without conditioning of the sorbent, so that the conditioning step was removed in this assay. It is worth mentioning that extraction without conditioning step is considered as an optional step for extraction of urine samples, but not being encouraged for plasma. Our results demonstrated that it can be applied to the plasma analysis without sacrificing the assay recovery and reproducibility. Before extraction, the sample was mixed with an equal volume of acid solution to lower the pH enough to form protonated analytes, and also to release analytes from protein binding. Several acid solutions, including 2% and 10% formic acid, 0.4 M phosphoric acid (pH 2) and 0.6% (0.1 M) acetic acid were evaluated. It appeared that there was no significant difference between the tested acid solutions in terms of recovery, which indicated that the weak acid 0.1 M acetic acid (relative to other tested acid solutions) was strong enough to protonate compounds **1**–**3**. Therefore, 0.1 M acetic acid was chosen to acidify plasma as loading mix. After washing with 0.1 M acetic acid and 100% acetonitrile, an attempt to add a third washing step to elute out some basic but more polar impurities was not successful, because analyte breakthrough was observed with the third wash containing as little as 10% acetonitrile in 5% ammonium hydroxide solution. After rinsing, sample was eluted with methanol/30% ammonium hydroxide (95/5, v/v), equivalent to a final 1.5% ammonium hydroxide in the eluting solvent. The composition of the elution solvent is critical for the simultaneous determination of both compounds, because replacement of methanol with acetonitrile in the elution solvent significantly lowered the recovery of compound **2**. The stability of compounds **1** and **2** under the sample preparation conditions was tested and confirmed.

Extraction recovery and the effect of sample matrix on LC–MS/MS detection were evaluated for both analytes and the internal standard. Recovery of the extraction procedure was determined by comparing the absolute peak area of standard in human plasma prepared as per the described bioanalytical method, with that of post-spiked standard-control plasma extracted in the same manner and then spiked with a know amount of analytes. Five replicates were used for compounds **1** and **2** at each of the nominal concentrations of 0.2, 10 and 300 ng/mL, which generated 15 replicates of I.S. at 5 ng/mL working concentration. The mean extraction recoveries for compounds **1**–**3** were 79.9%, 83.2% and 84.4%, and coefficients of variation were $\leq$3.6%, $\leq$7.7% and 2.8%, respectively (Table 1). Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak area of the post-spiked standard to that of neat standard in reconstitution solvent. Less than 10% matrix suppression was observed (Table 1). Based on this result and the intra-day precision and accuracy values obtained from five different lots of plasma (Table 2), the matrix effect should not have a significant impact on assay performance.

The availability of automation and Oasis MCX SPE plate provided significant benefit on the sample throughput and efficient sample clean up. During the sample preparation, standards and internal standard, QCs and plasma samples were pipetted into 96-well plate by Packard MultiPORBE II liquid handling system. Once in the 96-well format, all liquid transfer steps were handled using TomTec Quadra 96 workstation, including sample loading, washing and eluting. The Oasis MCX cartridge stood out among other choices because of its high selectivity and low matrix effect. Our results proved that, in extracting basic drugs from human plasma, Oasis MCX gave reasonable recovery with limited variation without requiring a conditioning step. In addition, since Oasis MCX is a polymer based sorbent, there was no impact on performance if the cartridge runs dry during sample preparation.

Table 1
Extraction recoveries and matrix effects of compounds **1**–**3** (I.S.) in human plasma

Nominal concentration (ng/mL)	Mean peak area (n = 5) (pre-spiked)[a]	Mean peak area (n = 5) (post-spiked)[b]	Mean peak area (n = 5) (neat)[c]	Recovery[d] (%) [%CV]	Matrix effect[e] (%) [%CV]
Compound **1**					
0.2	5268	7272	7860	80.5 [3.4]	92.5 [4.6]
10	298200	431400	478600	76.8 [3.6]	90.1 [2.1]
300	8718000	11760000	12640000	82.4 [2.9]	93.0 [1.4]
Compound **2**					
0.2	3064	4168	3726	81.7 [7.7]	111.9 [4.9]
10	169200	228200	217000	82.4 [3.0]	105.2 [1.9]
300	4652000	6052000	6002000	85.4 [4.1]	100.8 [0.7]
I.S. (n = 15)					
5	135933	178133	197133	84.4 [2.8]	90.4 [4.0]

[a] Pre-spiked is the standard spiked into plasma before extraction.
[b] Post-spiked is the standard spiked in the extract of control plasma.
[c] Neat is the standard in reconstitution solvent.
[d] Calculated as [(mean pre-spiked peak area/mean post-spiked peak area) × 100]%.
[e] Calculated as [(mean post-spiked peak area/mean neat peak area) × 100]%.

Table 2
Intraday precision and accuracy for the determination of compounds **1** and **2** in five lots of control human plasma

Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Accuracy[a] (%)	Precision[b] (%CV)
Compound **1**			
0.050	0.051	102.1	4.9
0.10	0.099	98.8	4.4
0.20	0.19	95.1	2.3
0.50	0.49	97.3	2.4
2.0	1.97	98.3	1.8
10.0	10.4	104.0	1.4
20.0	20.1	100.3	1.0
100.0	104.6	104.6	0.4
300.0	299.0	99.7	0.7
400.0	399.6	99.9	1.3
Linear regression	$R = 0.998$; slope = 0.213; intercept = −0.0007		
Compound **2**			
0.10	0.103	103.0	9.5
0.20	0.187	93.5	4.1
0.50	0.504	100.7	3.2
2.0	2.05	102.6	5.0
10.0	10.6	105.6	4.5
20.0	20.4	102.1	8.5
100.0	101.9	101.9	9.4
300.0	281.8	93.9	3.5
400.0	387.4	96.9	9.6
Linear regression	$R = 0.999$; slope = 0.118; intercept = −0.0007		

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]% ($n = 5$).
[b] Expressed as coefficient of variation (%CV) based on peak area ratios ($n = 5$).

3.3. Specificity, sensitivity, precision and accuracy

The selectivity of the plasma assay was assessed in six different lots of human control plasma. No interfering peak was observed in the retention time window of the analytes and internal standard under the assay conditions.

An assessment of intra-day variability of the assay was conducted with five standard curves that were prepared in five different lots of control human plasma over their calibration ranges, 0.05–400 ng/mL for compound **1** and 0.1–400 ng/mL for **2**. The assay precision and accuracy data and linear regressions for both analytes are presented in Table 2. The intra-day assay precision, as measured by coefficient of variation (%CV), was less than 4.9% for compound **1** and less than 9.6% for **2** for each concentration on both calibration curves. Assay accuracy was found to be 95.1–104.6% of nominal for all compound **1** standards and 93.5–105.6% for compound **2** standards. The linear correlation coefficient values for both analytes were better than or equal to 0.998. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the analyte that can be analyzed with an accuracy of ±15% of the nominal value and a coefficient of variation (%CV) ≤15%. The LLOQ for **1** was found to be 0.05 ng/mL with accuracy 102.1% and precision (%CV) 4.9%. The LLOQ for **2** was found to be 0.1 ng/mL with accuracy 103.0% and precision 9.5%.

Table 3
Intraday and interday accuracy and precision of quality control (QC) samples of compounds **1** and **2** in human plasma

Compound **1**	Measured concentration (ng/mL)		
	LQC 0.15 (ng/mL)	MQC 10 (ng/mL)	HQC 200 (ng/mL)
Intraday			
Mean ($n = 5$)	0.154	10.90	213.8
Precision (%CV)[b]	2.1	4.7	5.0
Accuracy (%)[a]	102.5	109.0	106.9
Interday			
Mean ($n = 3$)	0.161	10.4	211.0
S.D.	0.007	0.3	10.0
Precision (%CV)[b]	4.6	3.3	4.6
Accuracy (%)[a]	107.5	104.3	105.6
Compound **2**	LQC 0.3 (ng/mL)	MQC 10 (ng/mL)	HQC 200 (ng/mL)
Intraday			
Mean ($n = 5$)	0.340	9.72	198.8
Precision (%CV)[b]	4.2	6.2	6.1
Accuracy (%)[a]	113.3	97.2	99.4
Interday			
Mean ($n = 3$)	0.333	10.9	200.0
S.D.	0.023	0.5	14.0
Precision (%CV)[b]	6.9	5.0	7.0
Accuracy (%)[a]	111.1	109.1	100.0

[a] Expressed as [(mean measured concentration/nominal concentration) × 100]%.
[b] Expressed as coefficient of variation (%CV) based on peak area ratios.

Table 4
Stability of the QC samples of compounds **1** and **2** in human plasma (unit: percent of initial values in Table 3)

Nominal concentration (ng/mL)	Three freeze–thaw cycles ($n = 3$)	Room temperature 4 h ($n = 3$)	Processed sample in autosampler 16 h ($n = 3$)
Compound **1**			
0.15	102.8	105.8	101.9
0.3	99.9	105.3	104.4
10.0	98.2	100.6	97.9
200.0	98.5	98.8	90.9
Compound **2**			
0.3	91.1	98.7	99.7
10.0	113.5	115.9	120.0
200.0	109.8	110.2	106.6

3.4. Evaluation of QC stability

Quality control (QC) samples containing **1** and **2** were prepared at concentrations of 0.15 (three times of LLOQ for compound **1**), 0.3 (three times of LLOQ for **2**), 10 and 200 ng/mL. The intraday and interday precision and accuracy for QC samples at each concentration are given in Table 3, and the intraday measured mean concentrations were used as initial values to evaluate the stability of the analytes.

The stability experiments were designed to test effects of freeze–thaw cycles, short-term storage at room temperature, and storage in autosampler during sample analysis. These experiments were performed as described in Section 2.7, and all stability results are summarized in Table 4. It indicated that three freeze–thaw cycles for QC samples had no significant effect to the concentration of analytes; the analytes can well tolerate room temperature for at least 4 h during sample preparation; and both analytes were stable in the autosampler at 5 °C for at least 16 h. Since the extracted sample may need to be refrigerated during analysis, re-injection reproducibility was demonstrated by comparing the results of the same five intraday-validation curves analyzed before and after storage at −20 °C for 7 days. The results showed that the reinjected replicates met the same precision and accuracy criteria as the originally injected samples (data not shown), and indicated that storing the extracted sample at −20 °C did not affect the quantitative determination of both **1** and **2** in plasma samples.

Due to the poor solubility of compound **1**, precipitation was observed in its 1 μg/mL stock solution in acetonitrile/H_2O (50/50, v/v) after storage at −20 °C. To solve this problem, the stock solutions and working solutions were prepared in acetonitrile/0.1% formic acid (50/50, v/v), and stored at 4 °C. Because of this acidic storage condition, the evaluation of working-stock stability became particularly important. This issue can be assessed by comparing the measured concentration of working stock with its desired nominal concentration. The calibration standard curve in this case was constructed with fresh-made working stock solutions. It has been demonstrated that both analytes **1** and **2** were stable under the described storage and working conditions for at least 6 months (data not shown).

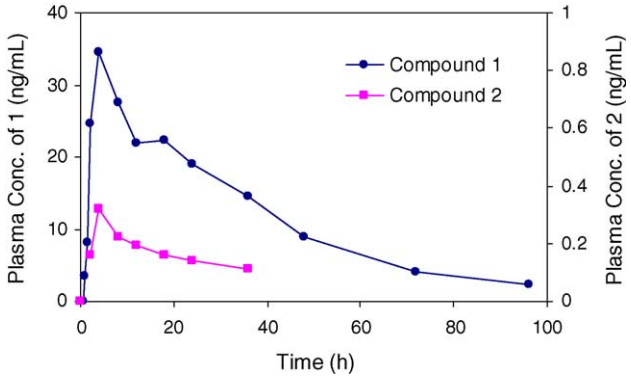

Fig. 4. Plasma concentration–time profile of compounds **1** and **2** from a healthy male subject #001 receiving a 12 mg single oral administration of compound **1**.

3.5. Application to clinical studies

The described method has been successfully applied to five clinical studies to determine plasma concentration levels of compounds **1** and/or **2** in support of pharmacokinetic analysis in Phase I clinical studies. Representative chromatograms of human clinical plasma samples obtained from a dosed subject are show in Fig. 3 (Panel C and D). A representative plasma concentration–time profile of compounds **1** and **2** in a healthy volunteer, subject #001, after receiving a single oral 12 mg dose of **1** is shown in Fig. 4. The active *N*-oxide metabolite, compound **2,** appears to be consistently less than 1% of parent **1** at all doses tested. The data suggest that **2** is a minor circulating metabolite in human.

4. Conclusion

A sensitive and automated assay combining mixed mode SPE and LC–MS/MS was developed and validated for the simultaneous determination of compound **1** and its active metabolite **2** in human plasma. The mixed mode SPE procedure provided a highly efficient sample clean up with excellent recoveries, without requiring a sorbent conditioning step. The combination of HPLC and MS/MS led to specificity and sensitivity of drug quantification with broad dynamic ranges. Automation and short HPLC run time increased throughput of sample analysis. The method has been successfully applied to the determination of plasma concentration levels of compounds **1** and **2** in support of pharmacokinetic studies in phase I clinical trails.

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