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Strategies on efficient method development of on-line extraction assays for determination of MK-0974 in human plasma and urine using turbulent-flow chromatography and tandem mass spectrometry

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Abstract

On-line extraction assays using cohesive high-turbulence liquid chromatography (HTLC) coupled with tandem mass spectrometer (MS/MS) have been developed for the determination of MK-0974 in human plasma and urine. In this report, a four-step strategy for efficient method development of an on-line extraction assay was discussed. Several challenges – namely extraction recovery, carryover and analyte loss to urine container – were addressed. The assay procedures included sample preparation on a Packard MultiPROBE II liquid-handling system, direct injection on a Cohesive Flux 2300 system, on-line extraction with a Cohesive C_{18} column (0.5 mm × 50 mm, 50 μm), HPLC separation on a FluophasePFP column (50 mm × 3 mm, 5 μm) under cohesive quick-elution mode and MS detection on a Sciex API4000 in multiple-reaction monitoring (MRM) mode using positive ionization and turbo ion-spray. Since 37–80% analyte loss was observed in urine QCs, 1% BSA was added to bring urine QC accuracy back to ∼100% of nominal. Because of the nature of BSA, the urine assay was established by adapting the plasma method. Thus, the two assays were able to be validated side-by-side, which reduced the validation time by approximately two-fold. The linear dynamic ranges were 0.5–500 and 1–1000 nM for the plasma and urine assays, respectively. Obtained from five standard curves constructed with five lots of human plasma or urine, the intra-day precision (%CV) was <3.14 and <2.62%, and the accuracy was 98.3–101.0 and 99.13–100.64% of nominal for plasma and urine assays, respectively. Both plasma and urine QC samples were stable when kept at room temperature for 4 h, at −70 °C for 3 weeks, or after three freeze–thaw cycles. Both assays gave reasonable relative recovery (>88.8%) and acceptable matrix effect (<15%). The carryover from the upper limit of quantification (ULOQ) was able to be controlled at <20% of lower limit of quantification (LLOQ).

Keywords: On-line extraction; Cohesive technology; High-turbulence liquid chromatograph (HTLC); Parallel validation; CGRP receptor antagonist; MK-0974

1. Introduction

The release of calcitonin gene-related peptide (CGRP) from trigeminal nerves plays a central role in the underlying pathophysiology of migraine [1–4]. MK-0974, *N*-[(3*R*,6*S*)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifuoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1H-imidazo-[4,5-B] pyridine-1-yl) piperidine-1-carboxaminde (Fig. 1), is an orally active drug candidate currently under development as a CGRP receptor antagonist for the treatment of migraine [5,6]. To support the clinical studies, developing sensitive and reliable bioanalytical assays to determine the concentration of MK-0974 in human plasma and urine are essential.

On-line extraction using cohesive high-turbulence liquid chromatography (HTLC) coupled with tandem mass spectrometry (MS/MS) has been widely used in the pharmaceutical industry to support drug discovery and development [7–15]. The crude biological fluid can be directly injected onto an extraction column packed with 30–60 μM spherical porous particles with high flow-rate under turbulent-flow conditions, through which a fast and efficient separation of the small molecule drug from the large plasma proteins will be achieved. The analytes and

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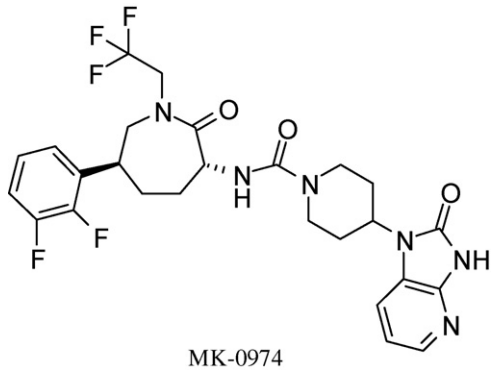
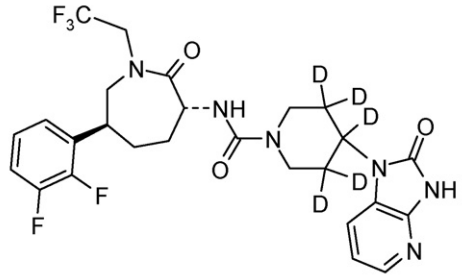


MK-0974



Internal standard (I.S.)

Fig. 1. Chemical structures of MK-0974 and its internal standard D5 MK-0974 (I.S.).

internal standard are retained on the extraction column, eluted and separated on the analytical column, and detected on mass spectrometry. The major challenges associated with this technology are timely method development, extraction recovery and carryover. A general practical strategy to address these challenges will benefit future method development.

For urine assays, the analyte loss to the container surface is getting more attention in bioanalytical method development. Addition of bovine serum albumin (BSA) and non-ionic surfactant Tween-20 is reported from different laboratories to resolve this issue for measurement of small-molecular drug candidates in urine [16–18]. As discussed in our previous publication [18], there are pros and cons in choosing different additives. BSA, as part of plasma protein, generally works well for the urine assay that is modified from the validated plasma assay. However, its effect appears to be compound-dependent [18]. In contrast, Tween-20 can effectively prevent analyte adsorption to the container surface, and addition of Tween-20 to the urine sample has been recommended for the assay that could washout Tween-20 during sample cleanup/extraction. In the case of on-line extraction, one major concern is whether the extraction column could retain the non-ionic surfactant Tween-20 and cause ion suppression when Tween-20 is eluted through the analytical column onto the MS. The discussion regarding the additive selection in an on-line extraction assay will provide guidance to the similar situation.

This report describes a four-step strategy used in developing on-line extraction HTLC-MS/MS assays under cohesive quick-elution mode for the determination of MK-0974 in human plasma and urine, with highlights on the challenges – namely extraction recovery, carryover and analyte loss to urine container – during method development. The side-by-side approach for efficient validation of plasma and urine assays is discussed. The assay precision, accuracy, QC stability, recovery and matrix effects are evaluated.

2. Experimental

2.1. Materials

MK-0974, internal standard and its N-oxide metabolite (Fig. 1) were synthesized as 99.8, 98.8 and 98.5% purity, respectively, at the Merck Research Laboratories, Merck & Co. (West Point, PA, USA). HPLC grade acetonitrile, laboratory grade formic acid (90%) and ACS grade acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade tetrahydrofuran and 35% bovine serum albumin (BSA) were obtained from Sigma–Aldrich (Milwaukee, WI, USA). Human control plasma (sodium heparin as anticoagulant) was purchased from Biological Specialty Co. (Colmar, PA, USA). Human control urine was collected from healthy volunteers. Water was purified by a Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA).

2.2. Instruments

A Cohesive Aria^{TM} 2300 system (Cohesive Technologies Inc., Franklin, MA, USA) was used for on-line extraction which included two quaternary Flux pumps, a valve module and a CTC HTS autosampler. A Packard MultiPROBE II HT EX automated liquid handling system (Meriden, CT, USA) was used to perform sample preparation. A Sciex API 4000 triple quadrupole mass spectrometer with a Sciex Turbo Ion Spray Interface (Sciex, Toronto, Canada) was used as a detector. The data were collected and processed through Analyst 1.4 software.

2.3. Chromatographic conditions

The on-line extraction was done using the dual column quick-elution mode on cohesive, and the system configuration during sample loading, transferring and eluting steps is illustrated in Fig. 2. The turbulent-flow extraction column, Cohesive C_{18} (0.5 mm × 50 mm, 50 μm) from Cohesive Technologies Inc. (Franklin, MA, USA), was used to isolate analytes from human plasma. The chromatographic separation was achieved on an analytical column, FluoPhase PFP, (50 mm × 3 mm, 5 μm) from Thermo-Hypersil Keystone under room temperature. The compartment of the autosampler was set at 5 °C. Four solutions, A: 0.1% formic acid (FA) in Milli-Q water; B: 0.1% FA in acetonitrile; C: 15% acetic acid in water; D: tetrahydrofuran/acetonitrile (90/10, v/v), were used as mobile phases. The turbulent-flow LC method on the cohesive system is listed in Table 1 and the total run time is about 6.5 min.

2.4. Mass spectrometry detection and calculation

A PE Sciex API 4000 triple-quadrupole mass spectrometer with a turbo-ionspray interface ionization source operated

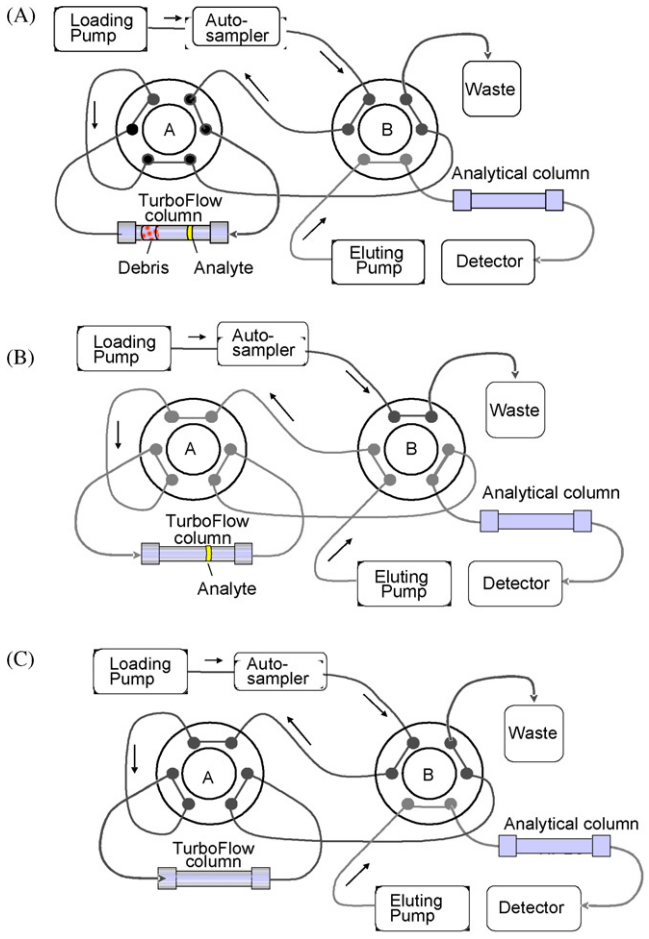


Fig. 2. Flow chart and valve configuration of cohesive instrument under quick-elution mode: (A) loading step—turbulent flow sweeps debris from sample matrix through HTLC extraction column while analyte was retained. (B) Transfer step—gradient mobile phase eluted analytes back out of HTLC extraction column to analytical column. (C) Eluting step—analytes were separated through eluting from analytical column to detector.

in a positive ion mode was used to detect the analytes. Precursor ions for MK-0974 and 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 567 → 219 for MK-0974 and m/z 572 → 224 for I.S. were selected for multiple reaction monitoring (MRM). The instrument setting was adjusted to maximize the response for the analyte and I.S., respectively, using *t*-shape infusion in the presence of mobile phase. A high voltage of 5 kV was applied to the sprayer. The turbo gas temperature was 550 °C and the auxiliary gas flow was 60 L/min. The flow settings of nebulizing gas (nitrogen), collision gas (nitrogen) and curtain gas (nitrogen) at the instrument were 55, 11 and 35 L/min, respectively. The optimized declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and entrance potential (EP) were 46, 33, 20 and 10 V for both MK-0974 and I.S. The dwell time was 150 ms for each compound. Both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total acquisition time on MS was 5 min. Peak area ratios were calculated using Analyst software version 1.4. Calibration curve was obtained by weighed ($1/x^2$) least squares linear regression of the peak area ratio of the analyte to the internal standard versus the nominal concentration (x) of analyte.

2.5. Calibration standards and quality control (QC) samples

Two MK-0974 stock solutions at 200 μM were prepared from two separate weighing and dissolved in acetonitrile/water (50/50, v/v). One set of analyte stock solutions was used to prepare calibration standards, and the other set was used to make QC samples. Working standards, containing MK-0974 at 2, 4, 20, 200, 400, 800, 1600, 2000, 3200 and 4000 nM, were prepared by serial dilutions of analyte stock solution with acetonitrile/water (50/50, v/v), and stored in amber glass vials at 4 °C. An 80 nM I.S. working solution was obtained by dilution of 200 μM I.S. stock solution in acetonitrile/water (50/50, v/v). Plasma or urine calibration standards were prepared daily by adding 50 μL of working standard, 50 μL of 80 nM I.S. and 100 uL of 15% acetic acid into 200 μL of control plasma or BSA containing urine to provide final concentrations of MK-0974 at 0.5, 1, 5, 50, 100, 200, 400 and 500 nM in plasma or 1, 5, 50, 100, 200, 500, 8000 and 1000 nM in urine, respectively. The plasma QC samples

Table 1
Cohesive on-line extraction HTLC/LC method under quick-elution mode

Step	Time (s)	Loading pump						SD	CD	eluting pump					
		Flow (mL/min)	Grad	%A	%B	%C	%D			Flow (mL/min)	Grad	%A	%B	%C	%D
Load	25	1.25	Step	100	–	–	–	L	←	0.5	Step	50	50	–	–
Load	5	1.25	Step	100	–	–	–	L	→	0.5	Step	50	50	–	–
Transfer	30	1.25	Step	–	10	90	–	E	→	0.5	Ramp	30	70	–	–
Elute	60	1.25	Step	–	10	90	–	L	→	0.5	Step	30	70	–	–
Clean	45	1.25	Step	–	–	–	100	L	→	0.5	Step	–	–	–	100
Clean	15	1.75	Step	–	–	–	100	E	→	0.5	Step	–	–	–	100
Clean	15	1.75	Step	–	–	–	100	L	←	0.5	Step	–	–	–	100
Clean	30	1.75	Step	10	90	–	–	L	←	0.5	Step	10	90	–	–
Clean	30	1.75	Step	10	90	–	–	E	→	0.5	Step	10	90	–	–
Equilibrate	135	1.25	Step	100	–	–	–	L	←	0.5	Step	50	50	–	–

Note: SD, Switching valve direction, in which "L" is loading direction and "E" is eluting direction. CD, Extraction column direction.

were prepared at 1.5, 20 and 400 nM MK-0974 in human control plasma, and the urine QCs were prepared at 3, 50 and 800 nM in human control urine that contained 1% BSA. QC aliquots were stored in a −70 °C freezer.

2.6. Sample preparation

Clinical samples/QCs were thawed at room temperature, mixed, and centrifuged at 4000 rpm (∼1300 × *g* RCF, relative centrifugal force), at 10 °C for 10 min. An aliquot of 200 μL sample was then transferred into a 2 mL 96-well deep-well plate (Matrix Technologies Corp., Hudson, NH, USA) using a Packard MultiPROBE II robotic liquid handler. An aliquot of 50 μL solvent (to match the volume of standards), 50 μL of 80 nM I.S. working solution and 100 μL of 15% acetic acid were sequentially added to each well. After vortex, the sample plate was centrifuged at 2000 RCF at 10 °C for 10 min, followed by direct injection of 10 μL prepared sample into Cohesive HTLC/LC–MS/MS system for sample on-line extraction and analysis.

2.7. Method validation

The side-by-side validations of plasma and urine assays were conducted through in-parallel sample preparation on Multi-Probe liquid handling system followed by in-tandem injection on HTLC-MS/MS in the same day. The selectivity of the assay was confirmed by processing control plasma/urine from six different lots. Intra-day precision and accuracy were determined by analyzing five sets of spiked standard samples in control plasma and urine, respectively. The final concentrations on the standard curves were 0.5, 1, 5, 50, 100, 200, 400 and 500 nM in the plasma assay and 1, 5, 50, 100, 200, 500 800 and 1000 nM in the urine assay. QC samples were analyzed after first freezing and thawing, and the calculated concentrations were considered as the initial values. The storage stability was evaluated after storage of QCs in the −70 °C freezer for 3 weeks. Freeze–thaw stability was evaluated using QC samples that went through three cycles of freezing and thawing, with at least 1-day storage at −70 °C between each thawing. Bench-top QC stability was tested following 4 h at room temperature and comparing the measured concentrations with their initial values. The stability of processed samples in the autosampler was assessed by comparing the results of QC samples analyzed at the end of the run (∼32 h in autosampler) with those analyzed at the beginning of the run. Reinjection reproducibility was demonstrated by comparing the results of the same five intra-day validation curves analyzed before and after storage at 4 °C for 5 days. In order to examine the dilution integrity, five replicates of 10-fold high QC (4000 nM in plasma or 8000 nM in urine) were diluted by 10-fold with the corresponding control matrix during sample preparation and analyzed on LC–MS/MS.

2.8. Extraction recovery and matrix effect

Extraction recovery was determined by comparing the peak areas of neat analyte solution after extraction to that of neat without going through the extraction column. The tested concentrations covered the dynamic ranges of both plasma and urine assays—0.5 (LLOQ for plasma), 1 nM (LLOQ for urine), 50 nM (middle concentration in both assays), 500 nM (ULOQ for plasma) and 1000 nM (ULOQ for urine) of MK-0974 and working concentration of 20 nM for internal standard. The recovery reported here only reflects the extraction of analyte from neat solution, not from the plasma or urine. Therefore, it can only serve as a relative reference value for the existing extraction method.

Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak area ($n = 5$ at each concentration) of plasma/urine samples to that of extracted neat standard at the same concentrations. The tested concentrations were 0.5, 50 and 500 nM for plasma assay and 1, 50 and 1000 nM for urine assay. Strictly speaking, the matrix effect observed in this experiment should reflect the combined differences of matrix and recovery between samples and neat solutions.

3. Results and discussion

3.1. Strategy for cohesive on-line extraction assay development

The on-line extraction HTLC-MS/MS was based on the dual column configuration, where the turbulent-flow extraction column retained the analytes of interest and washed away the matrix proteins and salts, and then analytes were subsequently transferred from the extraction column onto an analytical column using stronger mobile phase followed by final separation prior to MS detection.

To ensure efficient method development, a four-step strategy was applied.

The first step was evaluation of the conditions for mass spectrometry detection and chromatography on the analytical column under single column configuration, which is generally the same as most HPLC-MS/MS assay development with offline sample cleanup/extraction. The mass spectrometry conditions were set up by comparing the sensitivity under atmospheric pressure chemical ionization (APCI) versus turbo-ion spray (TIS) interface under positive versus negative ionization mode. TIS interface under positive ion mode was selected and the state file parameters were optimized to give the highest sensitivity for MK-0974 and I.S. The representative product ion mass spectra of the protonated MK-0974 and its internal standard are shown in Fig. 3. A number of analytical columns (such as Thermo Hypersil BDS C_{18}, Phenomenex Polar RP and Phenomenex Luna CN) were screened. FluophasePFP (50 mm × 3 mm, 5 μm) from Thermo-Hypersil Keystone exhibited the best peak shape and retention characteristics for MK-0974 when 60% acetonitrile in 0.1% formic acid (FA) aqueous solution was used as a mobile phase. Based on this information, FluoPhasePFP was selected as the analytical column and the gradient elution in HTLC method was tentatively chosen ramping from 50 to 70% acetonitrile which covered the optimal mobile phase strength (60% acetonitrile) if the initial mobile phase strength of 50% acetonitrile was strong enough to transfer the analyte from the

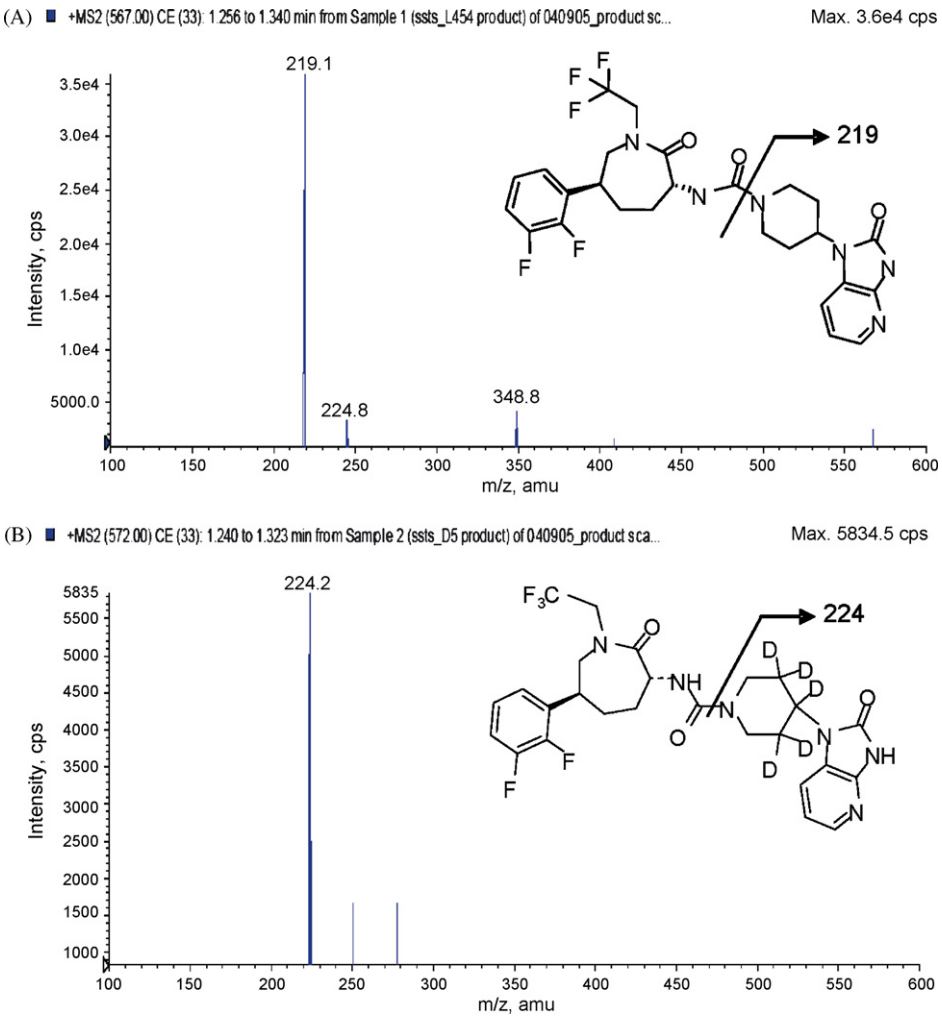


Fig. 3. Product ion mass spectra of the protonated molecules $(M+H)^+$ at *m/z* 567 and 572 for (A) MK-0974 and (B) I.S., respectively.

extraction column to the analytical column. Since the change of mobile phase pH (from 3 to 5) did not have significant impact on the chromatographic performance of MK-0974, pH 3 mobile phase was selected because it was conveniently available by using 0.1% formic acid.

The second step was a quick start with on-line extraction by selecting the basic elements of cohesive HTLC system. Cohesive quick-elution mode (Fig. 2), rather than focusing mode, was used because of its low carryover potential. The extraction column was compared and selected between cohesive HTLC C_{18} and cyclone at different available column dimensions, 0.5 mm × 50 mm versus 1.0 mm × 50 mm. HTLC C_{18} (0.5 mm × 50 mm) was chosen because it extracted MK-0974 with similar recovery but less carryover compared to cyclone (0.5 mm × 50 mm) while consuming less solvent compared to C_{18} with 1 mm × 50 mm dimension. The analytical column was Keystone FluoPhasePFP (50 mm × 3 mm, 5 μm), as indicated before. The mobile phase consideration was: (1) loading and eluting with mobile phases A and B to retain the analytes on the extraction column and analyze them on the analytical column; (2) extensive washing with mobile phases C and D to minimize the carryover and prolong the column lifetime. Along this line, a quick start on mobile phase selection was using (1) A: 0.1% formic acid in milliQ water and B: 0.1% formic acid in acetonitrile – the combination compatible with the analytical column conditions for MK-0974 as discussed in Step 1, (2) C: 15% acetic acid and D: 10% acetonitrile in tetrahydrofuran (THF) – the mobile phase combination that has been proven to significantly extend the HTLC extraction column life-time by washing away plasma proteins and lipids from HTLC extraction columns [19]. The HTLC method was set up as loading with aqueous mobile phase (100% A) to maximize analyte retention on the extraction column, transferring with 50% B (A/B = 50/50, v/v) to transfer analytes from the extraction column to the analytical column, and ramping from 50% B to 70% B (based on the test result from Step 1) within 5 min for a final separation prior to detection. With above key elements in place, the on-line extraction HTLC/LC–MS/MS assay was established.

The third step was to optimize the recovery by carefully choosing appropriate sample preparation, mobile phase composition and timing on loading and eluting steps. The sample preparation was designed considering the following factors: (1) acidify the sample to disturb the protein binding in plasma and give a more consistent recovery; (2) limit <15% acetonitrile

(introduced by adding standard and internal standard working solution) in the final sample plate to prevent analyte loss during the loading step and minimize protein precipitation when the samples were waiting for injection on the autosampler. Loading and eluting mobile phases were evaluated by connecting the extraction column to the mass spectrometer directly, injecting the neat MK-0974 solution on the extraction column, eluting with different strength mobile phases (stepwise change of mobile phase ratio A/B from 100/0 to 0/100 with 10% increment on B) and checking on analyte breakthrough. The result showed that MK-0974 was slightly leaking from the extraction column at 20% B and completely washed out at 40% B. Therefore, the strength of loading solvent at or below 10% B was safe to retain analyte and the eluting solvent at 50% should be high enough to transfer analyte from the extraction column to the analytical column. The timing of loading and transferring steps was minimized to shorten the run time, but ensure it was long enough to wash away debris and transfer the analyte, respectively.

The last step, also the most challenging step, was to minimize the carryover by optimizing the wash on the autosampler, extraction column, analytical column and switching valves. The needle alignment on the autosampler was carefully adjusted to minimize the dead volume at the injection valve. The needle wash solvents were selected: 5% acetonitrile in 0.1% FA aqueous solution as solvent 1 to wash out the proteins and salts, and 0.1% FA in 60% acetonitrile as solvent 2 to clean up the hydrophobic components including analyte and the internal standard. The washing program on the autosampler was optimized to completely clean up the protein from the syringe and injection valve with solvent 1 and then move to solvent 2. This was critical because the residue protein, if there was any, would be precipitated with solvent 2 and accumulated in the injection pathway to cause the carryover. The washes for the extraction column, analytical column and switching valve were programmed into the HTLC method using a combination of mobile phases A, B, C and D as cleaning solvents. Following the finalized cohesive HTLC/LC method (Table 1), the carryover, calculated as (peak area of solvent injection immediately after the upper limit of quantification/peak area of lower limit of quantification) × 100%, was less than 20% of the LLOQ for both plasma and urine assays that had 1000-fold dynamic range.

3.2. Effect of additives on the on-line extraction urine assay

During method development for the urine assay, the QC accuracy of MK-0974 ranged from 37 to 80% with a significant variation, which indicated adsorption of analyte to the container surface. A similar issue was discussed in our previous publication [18] and the effects of additives, particularly pros and cons of using Tween-20 versus bovine serum albumin (BSA), were discussed. Tween-20 was recommended for the basic/acidic drug candidates that can be potentially extracted using ion (cation or anion) exchange techniques, such as Oasis MCX or MAX (mixed mode of reversed phase and ion exchange) SPE products, while BSA was more suitable for liquid–liquid extraction, reversed-phase SPE and on-line extraction. In agreement with the above statement, addition of 0.2% Tween-20 caused more than 80% ion suppression for MS/MS detection of MK-0974, because the extraction column C_{18} extracted the non-ionic surfactant Tween-20 along with analytes and eluted them through the analytical column to the mass spectrometer. In contrast, addition of BSA was not a problem and served as a better choice for the urine assay using HTLC C_{18} on-line extraction. The amount of BSA addition was tested at different levels (0.25, 0.5, 0.75, 1, 1.25 and 1.5% of urine volume), and the QC accuracy reached plateau (∼100%) for all QC levels at around 0.75% BSA. To ensure good QC recovery and considering the difficulty of BSA handling, addition of 1% BSA was used for the clinical sample collection. BSA can be added before (the situation used for preparation of standard and QC samples) or after (the situation similar to sample collection at the clinical site) spiking analyte into control urine (data not shown). Because of the nature of BSA, the on-line extraction urine assay was easily established by simply adapting the plasma assay procedures.

3.3. Side-by-side validation of plasma and urine assays

The validations of both plasma and urine assays were conducted within the same day through in-parallel sample preparation on MultiPROBE II followed by in-tandem sample injection on HTLC/LC–MS/MS analysis. This side-by-side validation approach reduced the validation time by approximately two-fold, which was particularly useful to support the First-In-Men (FIM) study where the method development and validation timelines were compressed.

The selectivity of the plasma and urine assays was assessed in six different lots of human control plasma or urine. Representative extracted ion chromatograms of a control single blank and a plasma/urine standard at the lower limit of quantification (LLOQ) of MK-0974—0.5 nM for plasma assay/1 nM for urine assay, are shown in Fig. 4A and B (plasma), and Fig. 5A and B (urine). No interfering peak was observed in the retention time window of MK-0974 and the internal standard under the assay conditions. MK-0974 and its N-oxide metabolite were baseline separated chromatographically (0.5 min apart), which eliminated the potential interference caused by in-source fragmentation (data not shown).

The intra-day variability of plasma and urine assays was evaluated with five different lots of control human plasma/urine spiked with MK-0974 over the calibration range of 0.5–500 nM for the plasma assay and 1–1000 nM for the urine assay. The LLOQ were 0.5 and 1 nM for the plasma and urine assays, respectively. A weighted ($1/x^2$, where x is standard concentration of analyte) least-squares regression calibration curve was constructed by plotting the peak area ratios of analyte to internal standard versus standard concentrations. The intra-day precisions (%CV) were <3.14 and <2.62% for the plasma and urine assays, respectively, at each concentration of MK-0974 on the calibration curves. Assay accuracy was found to be 98.3–101.0 and 99.13–100.64% of nominal MK-0974 concentrations for plasma and urine assays, respectively (Table 2).

Quality control samples containing MK-0974 were prepared at 1.5 (3 times the concentration of the LLOQ), 20 and 400 nM

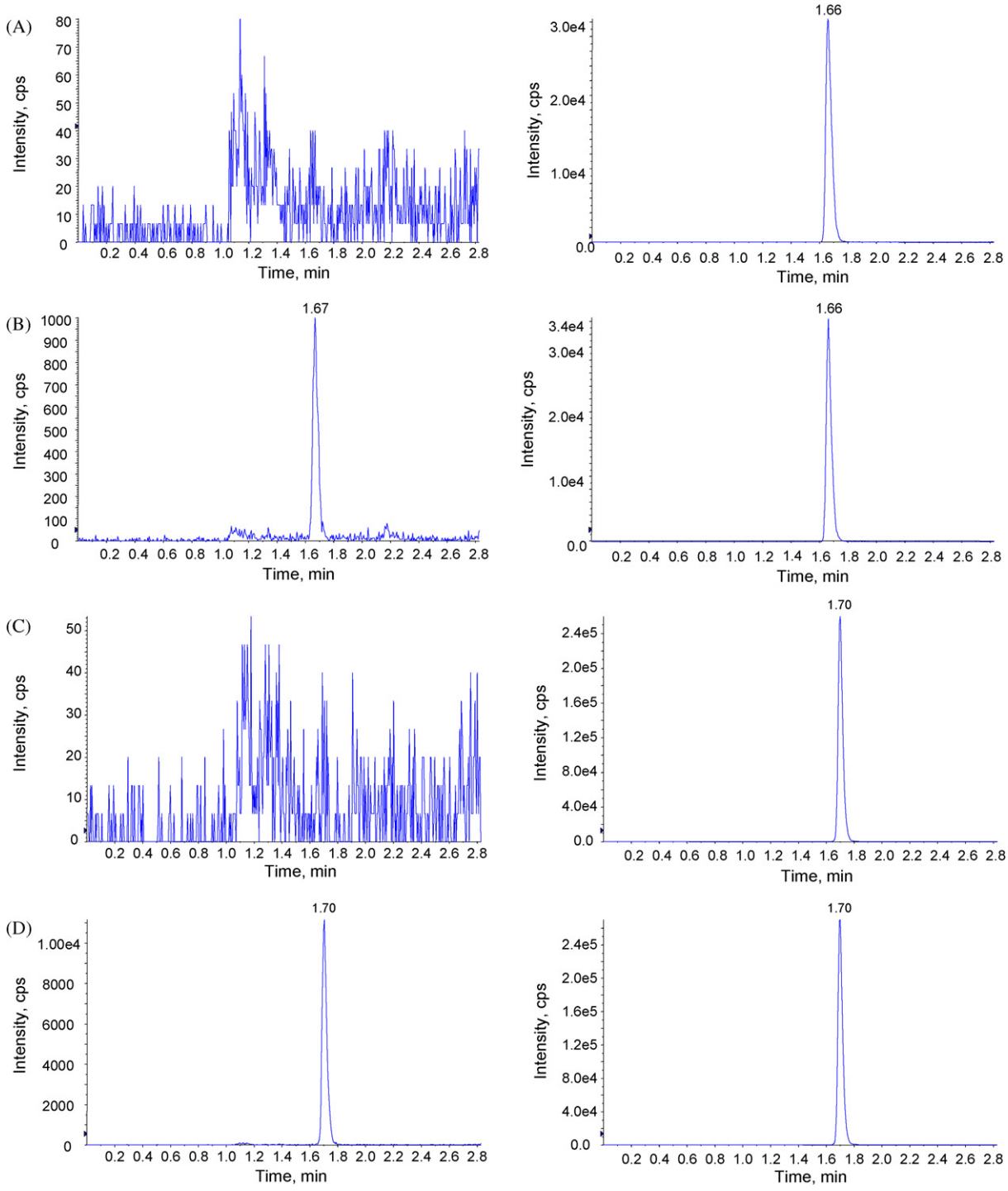


Fig. 4. Representative extracted ion chromatograms (*m/z* 567 → 219 for MK-0974 and *m/z* 572 → 224 for I.S.) from human plasma: (A) single blank; (B) 0.5 nM standard—lower limit of quantification (LLOQ); (C) pre-dose clinical sample from subject #002 and (D) 1 h post-dose clinical sample from subject #002 following a 2 mg single oral dose of MK-0974, corresponding to 5.64 nM MK-0974 (left panel: MK-0974 and right panel: I.S.).

for plasma assay, and 3, 50 and 800 nM for urine assay. In order to demonstrate the ability to dilute samples above the upper limit of the standard curve, 10× HQC samples, 4000 nM in plasma and 8000 nM in urine, were prepared and analyzed with 10-fold dilution. The results of the initial analyses ($n = 5$) of these samples are shown in Table 3, and the inter-day reproducibility and accuracy are showed in Table 4.

The QC stability tests for both plasma and urine assays were conducted to evaluate the effects of freeze–thaw cycles, exposure at room temperature, storage at −70 °C and waiting on autosampler for sample analysis. These experiments were performed as described in Section 2. The results, summarized in Table 5, indicated that there were no stability issues for both plasma and urine assays under the tested conditions.

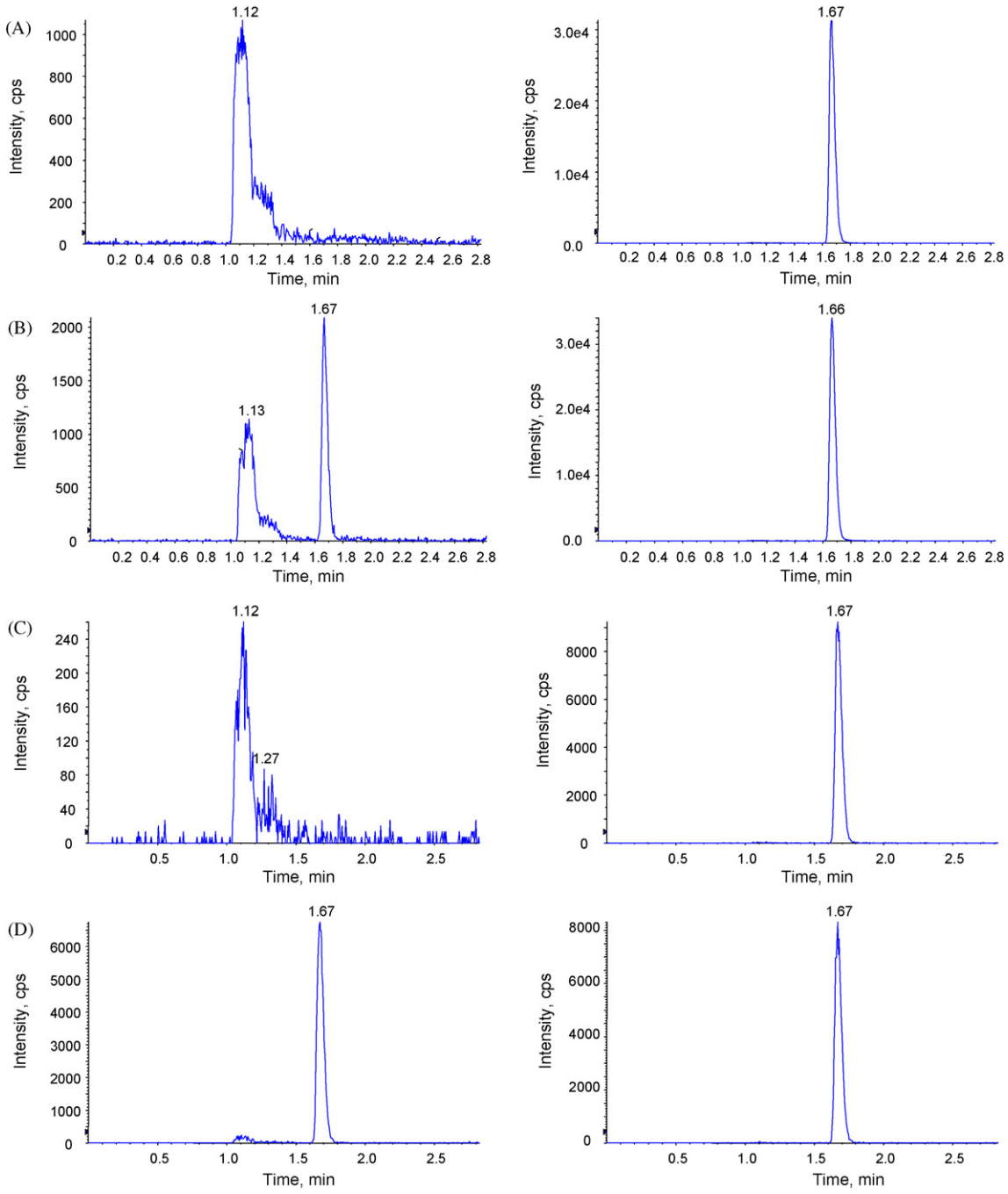


Fig. 5. Representative extracted ion chromatograms (*m/z* 567 → 219 for MK-0974 and *m/z* 572 → 224 for I.S.) from human urine: (A) single blank; (B) 1 nM standard—LLOQ; (C) pre-dose clinical sample from subject 002 and (D) 1–4 h post-dose clinical sample from subject #002 following a 2 mg single oral dose of MK-0974, corresponding to 12.81 nM MK-0974 (left panel: MK-0974 and right panel: I.S.).

Extraction recovery and matrix effect were evaluated for both plasma and urine assays. Recovery of the extraction procedures was determined by comparing the absolute peak areas of neat analyte after extraction to that of neat without going through extraction column (analytical column only). The tested concentrations covered the dynamic ranges of both plasma and urine assays—0.5 nM (LLOQ for plasma), 1 nM (LLOQ for urine), 50, 500 nM (ULOQ for plasma) and 1000 nM (ULOQ for urine) of MK-0974 and working concentration of 20 nM internal standard. The mean recoveries were 88.8–96.2 and 93.5% for MK-0974 and I.S., respectively (Table 6). Since the recovery reported here only reflected the extraction of analyte from neat solution, not from plasma or urine, it can only serve as a reference value for the existing extraction method. Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak area of plasma (at 0.5, 50 and 500 nM) or urine (at 1, 50 and 500 nM) samples after extraction to that of neat standard after extraction. Strictly speaking, this value

Table 2
Precision and accuracy for the determination of MK-0974 in five lots of control human biological matrix (plasma or urine)

Matrix	Nominal concentration (nM)	Mean concentration[a] (nM) ($n=5$)	Accuracy[b] (%)	Precision[c] (%CV)
Plasma	0.50	0.50	100.0	1.21
	1.00	1.01	101.0	3.14
	5.00	5.01	100.2	2.81
	50.00	50.41	100.8	1.21
	100.00	100.91	100.9	2.09
	200.00	199.03	99.5	1.89
	400.00	393.22	98.3	1.54
	500.00	498.69	99.7	1.28
Urine	1.00	1.00	100.00	2.62
	5.00	4.97	99.40	0.75
	50.00	50.32	100.64	1.53
	100.00	100.64	100.64	1.87
	200.00	200.11	100.06	0.86
	500.00	502.11	100.42	1.46
	800.00	797.43	99.68	0.38
	1000.00	991.30	99.13	1.63

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicated values at each concentration.
[b] Expressed as [(mean calculated concentration)/(nominal concentration) × 100] (%).
[c] Expressed as coefficient of variation (%CV) based on peak area ratios.

Table 3
Initial quality control sample analysis and dilution integrity of human plasma and urine QCs containing MK-0974

Matrix	Nominal concentration (nM)	Mean concentration (nM)	Accuracy[a] (%)	Precision[b] (%CV)
Plasma ($n=5$)	1.50	1.51	100.67	2.65
	20.00	20.53	102.65	1.07
	400.00	404.09	101.02	1.67
	4000.00 (10-fold dilution)	4092.66	102.32	0.95
Urine ($n=5$)	3.00	3.01	100.33	1.66
	50.00	50.52	101.04	0.99
	800.00	784.60	98.08	1.13
	8000.00 (10-fold dilution)	7856.66	98.21	2.37

[a] Expressed as [(mean calculated concentration)/(nominal concentration) × 100] (%).
[b] Expressed as coefficient of variation (%CV).

Table 4
Quality control (QCs) of MK-0974 during daily plasma and urine sample analysis

Matrix	Nominal concentration (nM)	Mean concentration (nM)	Accuracy[a] (%)	Precision[b] (%CV)
Plasma ($n=4$)[c]	1.50	1.48	98.67	4.73
	20.00	19.91	99.55	5.37
	400.00	386.17	96.54	4.40
Urine ($n=4$)[d]	3.00	3.29	109.67	4.56
	50.00	54.89	109.78	3.72
	800.00	894.91	111.86	4.27

[a] Expressed as [(mean calculated concentration)/(nominal concentration) × 100] (%).
[b] Expressed as coefficient of variation (%CV).
[c] $n=4$ runs, within each run, there were 3 replicates of QCs at each concentration.
[d] $n=4$ runs, within each run, there were 2 replicates of QCs at each concentration.

reflected the combination of matrix effect and recovery difference between samples and neat solutions. As a result, less than 15% of ion suppression/enhancement was observed (Table 6). Referring to the intra-day precision and accuracy obtained from five different lots of plasma and urine, the observed absolute matrix effect did not have any significant impact on the assay performance.

3.4. Applications to clinical studies

Representative chromatograms of human clinical plasma and urine samples obtained from a subject dosed at 2 mg MK-0974 are shown in Fig. 4C and D (plasma), and Fig. 5C and D (urine). The measured MK-0974 concentrations in plasma and urine were within the corresponding dynamic ranges, otherwise, appropriate dilution was performed. The plasma assay was applied to the First-In-Man study to determine the concentration of MK-0974 within a dose range of 2–80 mg in support of pharmacokinetics analysis. At the dose level above 80 mg, the diastereomer (confirmed by chiral assay) of MK-0974 was observed in the post-dose plasma samples, and thus the assay was modified accordingly. The lower-dose samples were double checked with the modified assay and the concentration of

Table 5
Stability of MK-0974 QCs in human plasma and urine

Matrix	Nominal concentration (nM)	3 F/T[a] [%CV] (%)	RT-4h[b] [%CV] (%)	AS-32h[c] [%CV] (%)	3 weeks[d] at −70 °C
Plasma ($n=3$)	1.50	98.90 [3.16]	103.53 [1.61]	100.13 [2.07]	105.30 [2.27]
	20.00	99.45 [1.44]	101.17 [2.13]	99.06 [0.84]	102.08 [0.28]
	400.00	98.61 [0.76]	100.80 [1.99]	98.86 [1.32]	101.30 [0.74]
Urine ($n=3$)	3.00	100.78 [2.95]	102.77 [2.29]	99.80 [1.65]	100.55 [1.63]
	50.00	101.06 [0.59]	103.99 [1.14]	100.49 [1.62]	101.50 [1.78]
	800.00	99.41 [1.31]	102.15 [0.82]	98.77 [0.86]	101.82 [0.53]

[a] Expressed as [(mean concentration after 3 freeze/thaw cycles)/(initial mean concentration in Table 3) × 100] (%).
[b] Expressed as [(mean concentration after room temperature for 4 h)/(initial mean concentration) × 100] (%).
[c] Expressed as [(mean concentration after being in autosampler for 32 h)/(initial mean concentration) × 100] (%).
[d] Expressed as [(mean concentration after storage at −70 °C for 3 weeks)/(initial mean concentration) × 100] (%).

Table 6
Relative extraction recovery and matrix effects on ionization in human plasma and urine assays

Matrix	Analyte	Nominal concentration (nM)	%CV on peak area after extraction ($n=3$)	Mean relative recovery (%)[a] ($n=5$)	Mean matrix effect (%)[b] ($n=5$)
Plasma	MK-0974	0.5	5.74	88.8	93.3
		50	1.55	96.2	86.9
		500	1.26	90.5	88.1
	I.S.	20	≤2.52[c]	93.5	87.4
Urine	MK-0974	1.00	5.77	91.1	114.1
		50.00	2.20	96.2	109.6
		1000.00	2.44	89.5	101.8
	I.S.	20	≤3.45[c]	93.5	108.7

[a] Expressed as [(mean peak area of neat standard after extraction/mean peak area of neat standard without extraction column) × 100] (%).
[b] Expressed as [(mean peak area of MK-0974 in plasma or urine after extraction/mean peak area of neat standard after extraction) × 100] (%).
[c] The highest %CV observed for I.S. under three concentrations of MK-0974.

diastereomer was negligible. The investigation on the chiral integrity of MK-0974 and modification of the plasma assay will be discussed in details elsewhere. No detectable diastereomer of MK-0974 was observed in urine samples across all dose levels. The described urine assay has been used to support multiple studies at the selected dose levels up to 800 mg of MK-0974.

4. Conclusion

On-line extraction coupled with liquid chromatography/tandem mass spectrometry (HTLC/LC–MS/MS) assays for the determination of MK-0974 in human plasma and urine has been developed and validated to support pharmacokinetic studies. The assays were fully automated using a Packard MultiPROBE II robotics system to handle sample preparation and a Cohesive Flux 2300 system coupled with a PE Sciex API 4000 for sample analysis. The linear calibration ranges were 0.5–500 and 1–1000 nM for plasma and urine assays, respectively. The assay precision and accuracy, and QC stability met the FDA guidance [20] requirement. Although the reported plasma assay was limited to measure the dose level up to 80 mg MK-0974 because of the observation of diastereomer in the post higher-dose plasma samples (the modified plasma assay and validation result will be discussed elsewhere), we believe that the general strategies presented here on efficient method development and resolving challenges, namely extraction recovery, carryover and analyte loss to urine container, will serve as a helpful tool for future method development.

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