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Elimination of diastereomer interference to determine Telcagepant (MK-0974) in human plasma using on-line turbulent-flow technology and off-line solid-phase extraction coupled with liquid chromatography/tandem mass spectrometry

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ABSTRACT

To eliminate the diastereomer interference on Telcagepant (MK-0974) determination during clinical study support, on-line high turbulent-flow liquid chromatography (HTLC) methods, HTLC-A and HTLC-B that covered dynamic range of 0.5–500 nM and 5–5000 nM, respectively, were developed. To meet the requirement of rapid assay transfer among multiple laboratories and analysts, a solid-phase extraction (SPE) assay was derived from the existing HTLC-B assay under the same dynamic range. The on-line HTLC assays were achieved through direct injection of plasma samples, extraction of analyte with a Cohesive C_{18} column (50 mm × 0.5 mm, 50 μm), followed by HPLC separation on a FluoPhase RP column (100 mm × 2.1 mm, 5 μm) and MS/MS detection. The off-line SPE assay used Waters Oasis®HLB μElution plate to extract the analytes from plasma matrix before injecting on a FluoPhase RP column (150 mm × 2.1 mm, 5 μm) for LC–MS/MS analysis. Under both on-line and off-line assay conditions, the diastereomer 1c was chromatographically separated from MK-0974. Cross-validation with the pooled samples demonstrated that both on-line and off-line assays provided comparable data with a difference of <2.6%. The assays were proved to be specific, accurate and reliable, and have been used to support multiple clinical studies. The pros and cons of on-line and off-line assays with regard to man power involved in sample preparation, total analysis time, carryover, cost efficiency, and the requirement for assay transfer are discussed.

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1. Introduction

Telcagepant (MK-0974 in Fig. 1) is a novel oral calcitonin gene-related peptide (CGRP) receptor antagonist being developed for the acute treatment of migraine [1–3]. To support the clinical studies, it was critical to develop a reliable bioanalytical assay to determine the concentration of MK-0974 in human plasma. An on-line extraction assay using Cohesive high-turbulence liquid chromatography (HTLC) coupled with tandem mass spectrometry (MS/MS) was developed before first-in-man to support MK-0974 clinical trials within the dose range of 2–80 mg [4]. However, at the dose level above 80 mg, interference was observed in the post-dose sample analysis [4]. The follow-up study confirmed that a diastereomer (1c in Fig. 1) of MK-0974 was the source of interference, and ruled out the possibility of chiral inversion at azepinone-6 carbon [5]. Since 1c was about 475-fold less potent than MK-0974 according to the *in vitro* CGRP binding data, and its exposure was ∼5% of MK-0974s [5], according to FDA's guidance for industry safety testing of drug metabolites, it was not necessary to determine the plasma level of 1c in pharmacokinetic studies. On the other hand, since the half-life of 1c was longer than that of MK-0974 [5], the interference became more significant at the later time points at higher dose levels due to accumulation; and therefore, elimination of diastereomer interference was critical for continued clinical study support of MK-0974.

The assay modification was conducted at different stages. As clinical trial proceeded to higher doses, the assay was quickly modified to separate MK-0974 from the interference of its diastereomer without changing the dynamic range (0.5–500 nM) of MK-0974; the modified assay is referred as HTLC-A. Once the clinical dose level was determined, the assay was adjusted to a higher dynamic range 5–5000 nM, referred as HTLC-B. As the program progressed, more internal and external contract laboratory support was required because of the large number of samples in late phase development. Applying the reversed-phase sorbent, used in the HTLC extraction column, an off-line solid-phase extraction (SPE) assay was developed to accommodate assay transfer between laboratories and support from multiple analysts.

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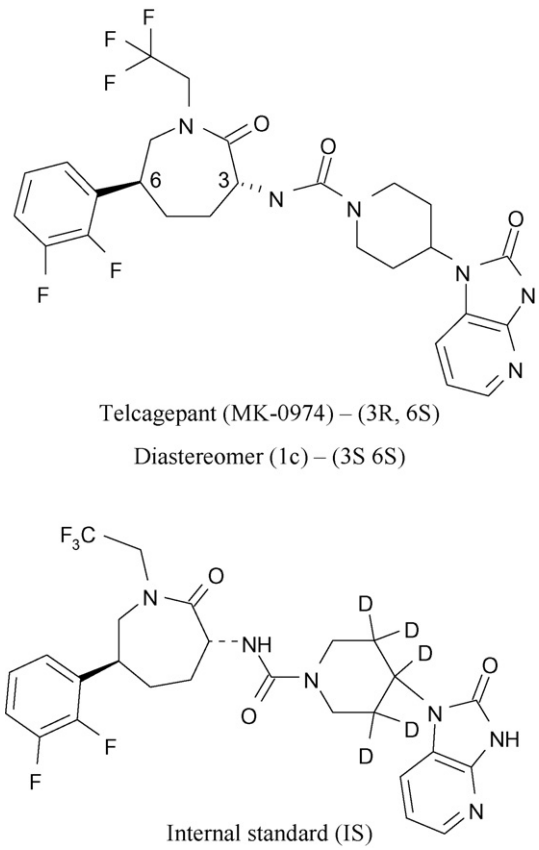

Fig. 1. Chemical structures of Telcagepant (MK-0974), its diastereomer (1c), and its internal standard D5 MK-0974 (IS).

This report highlights the results from both the Cohesive on-line HTLC extraction and off-line solid-phase extraction in determination of MK-0974 in human plasma. The pros and cons of these two technologies are compared and discussed. Since Cohesive on-line extraction and SPE off-line extraction have been widely used in the diagnosis laboratories and pharmaceutical industries, the method comparison shared in this report will provide helpful information for others to consider before starting any method development.

2. Experimental

2.1. Materials

MK-0974, its diastereomer (1c) and internal standard (IS, Fig. 1) were synthesized at the Merck Research Laboratories, Merck & Co. (West Point, PA). HPLC grade acetonitrile, laboratory grade formic acid (90%) and ACS grade acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade tetrahydrofuran was obtained from Sigma–Aldrich (Milwaukee, WI, 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). HTLC extraction column, Cohesive C_{18} (50 mm × 0.5 mm, 50 μm), was purchased from Cohesive Technologies Inc. (Franklin, MA, USA). Analytical columns, FluoPhase RP (100 mm × 2.1 mm, 5 μm) and FluoPhase RP (150 mm × 2.1 mm, 5 μm), were purchased from Thermo Fisher Scientific (Bellefonte, PA, USA). Oasis®HLB μElution plate (96-well) was purchased from Waters Corporation (Milford, MA, USA) and used for solid-phase extraction (SPE) of plasma samples.

2.2. Instruments

A Packard MultiPROBE II HT EX automated liquid handling system (Meriden, CT, USA) was used to perform sample preparation. A Cohesive Aria™ 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. For off-line solid-phase extraction assay, a TomTec Quadra-96 workstation (Model 320, Hamden, CT, USA) was used to perform automated SPE process, and a LC micro-pump (Series 200 from Perkin-Elmer, Ontario, Canada) coupled with a 96-well plate autosampler (HTS PAL System from Leap Technology, Carrboro, NC, USA) were used to perform high-performance liquid chromatographic (HPLC) separation after SPE extraction. A Sciex API4000 or API3000 triple-quadrupole mass spectrometer with a Sciex Turbo Ion Spray Interface (Sciex, Toronto, Canada) was used as a detector for on-line and off-line assays. The data were collected and processed through Analyst 1.4 software.

2.3. Chromatographic conditions

The on-line extraction (for both HTLC-A and HTLC-B) was conducted using the dual column quick-elution mode on Cohesive, and the system configuration during sample loading, transferring and eluting steps is illustrated in the previously published report [4] with modifications. The turbulent-flow extraction column, Cohesive C_{18} (50 mm × 0.5 mm, 50 μm), was used to isolate analytes from human plasma with a 10 μL injection volume. The chromatographic separation was achieved on an analytical column, FluoPhase RP (100 mm × 2.1 mm, 5 μm), at 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. This method adequately separated the diastereomer from MK-0974.

The samples for off-line SPE extraction were chromatographed on a FluoPhase RP (150 mm × 2.1 mm, 5 μm) column from Thermo-Hypersil Keystone with a 10 μL sample injection. The mobile phase was composed of acetonitrile and 0.1% formic acid aqueous solution at a ratio of 60/40 (v/v), and its flow rate was 0.2 mL/min. The column was maintained at room temperature and the autosampler was set at 5 °C.

2.4. Mass spectrometry detection and calculation

A PE Sciex API4000 triple-quadrupole mass spectrometer with a turbo-ionspray interface ionization source operated in the positive ion mode was used to quantitate MK-0974 at the dynamic range 0.5–500 nM for HTLC-A assay; while an API3000 with the same ionization mode was used for HTLC-B and SPE assays at the dynamic range of 5–5000 nM. The ion pairs (precursor ion → product ion) m/z 567 → 219 for MK-0974 and m/z 572 → 224 for IS were selected for multiple reaction monitoring (MRM). The instrument settings adopted the conditions published previously, including gas flow, turbo gas temperature, electric voltages, Analyst software, calculation, etc., on API4000 [4] and API3000 [5], respectively. The dwell time was 150 ms for MK-0974 and its IS. The total acquisition time for each injection on MS was 10 and 5 min for on-line and off-line assays, respectively.

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) for preparing calibration standards and QC samples, respec-

Table 1
HTLC on-line extraction HTLC/LC method under quick-elution mode (for both HTLC-A and HTLC-B).

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	←	0.2	Step	50	50	–	–
Load	5	1.25	Step	100	–	–	–	L	→	0.2	Step	50	50	–	–
Elute	120	1.25	Step	–	10	90	–	E	→	0.2	Ramp	30	70	–	–
Elute	60	1.25	Step	–	10	90	–	L	→	0.2	Step	30	70	–	–
Clean	90	1.25	Step	–	–	–	100	L	→	0.2	Step	–	–	–	100
Clean	30	1.75	Step	–	–	–	100	E	→	0.2	Step	–	–	–	100
Clean	30	1.75	Step	–	–	–	100	L	←	0.2	Step	–	–	–	100
Clean	30	1.75	Step	10	90	–	–	L	←	0.2	Step	10	90	–	–
Clean	30	1.75	Step	10	90	–	–	E	→	0.2	Step	10	90	–	–
Equilibrate	180	1.25	Step	100	–	–	–	L	←	0.2	Step	50	50	–	–

Note: SD, switching valve direction, in which, "L" is loading direction, and "E" is eluting direction; CD: extraction column direction.
Mobile phases: (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).

tively. Working standards of MK-0974 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. Plasma calibration standards were prepared daily by mixing 50 μL of working standard, 50 μL of 80 nM IS and 100 μL of 15% acetic acid with 200 μL of control plasma to provide final plasma MK-0974 concentrations at 0.5, 1, 5, 50, 100, 200, 400 and 500 nM for HTLC-A; or by mixing 50 μL of working standard, 50 μL of 80 nM IS, 100 μL of 15% acetic acid and 150 μL of Milli-Q water with 50 μL of control plasma to provide final plasma MK-0974 concentrations at 5, 10, 20, 200, 800, 2000, 4000 and 5000 nM for HTLC-B and SPE assays.

The QC samples were prepared at 1.5, 20 and 400 nM MK-0974 in human control plasma for HTLC-A, and at 15, 400 and 4000 for HTLC-B and SPE assays. All 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. For HTLC-A assay, an aliquot of 200 μL sample was then transferred into a 2-mL 96-well deep-well plate 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 IS working solution and 100 μL of 15% acetic acid was sequentially added to each well. This acidified sample mix was vortexed and centrifuged at 2000 RCF at 10 °C for 10 min, followed by direct injection of 10 μL supernatant into Cohesive HTLC/LC–MS/MS system.

For HTLC-B assay, the same procedures were followed, except using 50 μL of plasma sample and 150 μL of Milli-Q water to replace the 200 μL plasma in HTLC-A.

For off-line SPE extraction assay, the acidified sample mix was prepared in the same way as that in HTLC-B, and subsequently loaded onto the Oasis®HLB μElution 96-well SPE plate using TomTec Quadra-96 under applied vacuum. The SPE plate was preconditioned with 200 μL of acetonitrile and 200 μL of 0.1 M acetic acid sequentially. After loading, the sample wells were washed with 400 μL of 0.1 M acetic acid, followed by 400 μL of 10% acetonitrile. The analytes were eluted with 2× 0.1 mL of 100% acetonitrile into a new 1-mL 96-well deep-well collection plate. 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 into 200 μL of acetonitrile/water (50/50, v/v), and 10 μL of the sample were injected for LC–MS/MS analysis.

2.7. Method validation

The selectivity of the assay was confirmed by processing control plasma from six different lots. Intra-day precision and accuracy were determined by analyzing five standard curves, each prepared in a different lot of control plasma. Assay accuracy was calculated from a least-squares regression curve constructed using all five replicate values at each concentration, and the intra-day precision (%CV) was calculated from the peak area ratio of MK-0974 versus IS for each concentration used to construct the standard curve. Initial measurement of QC samples for different dynamic ranges were performed after the first freezing and thawing, and the measured concentrations were considered as the initial values. 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. Room temperature 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 with those analyzed at the beginning of the run. Re-injection stability 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. The long-term storage stability was evaluated after storage of QCs in a −70 °C freezer for a long period of time, and analyzed along with freshly prepared standards. In order to examine the dilution integrity, five replicates of a plasma sample with a concentration 10-fold higher than the high QC concentration were diluted 10-fold with the control matrix during sample preparation and analyzed on LC–MS/MS.

2.8. Extraction recovery and matrix effect

The extraction recovery and matrix effect were evaluated to cover the corresponding dynamic range of each assay at the MK-0974 concentrations of 0.5, 50 and 500 nM with an IS concentration of 20 nM for HTLC-A, and 5, 200 and 5000 nM with an IS concentration of 80 nM for HTLC-B and SPE. The data were reported as a mean of five replicate measurements at each concentration level.

For on-line extraction assay, the extraction recovery was determined by comparing the peak areas of neat analyte solution after extraction to that of neat solution with the extraction column bypassed. Matrix enhancement/suppression of ionization in the on-line assay was evaluated by comparing the absolute peak area of plasma samples to that of extracted neat standard at the same concentrations.

For off-line SPE extraction assay, the extraction recovery was determined by comparing the absolute peak areas of the pre-spiked samples with post-spiked samples. The pre-spiked samples were prepared by the extraction procedures described in Section 2.6, and the post-spiked samples were prepared by extracting drug-free control plasma and spiking with working stocks containing MK-0974 and IS after extraction. The matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas

of the post-spiked samples with neat standards at the appropriate concentration in reconstitution solvent.

3. Results and discussion

3.1. Development of on-line and off-line extraction assays to eliminate the interference from the diastereomer of MK-0974

As reported previously [4], an on-line HTLC assay was developed to support MK-0974 clinical studies before the first-in-man study. During sample analysis, a diastereomer (1c) of MK-0974 was observed and confirmed in the post-dose human plasma sample at the dose level above 80 mg during the first human study [4,5]. To continue supporting the clinical studies at high dose levels, an assay modification was necessary to eliminate the interference. Since mass spectrometric separation of the diastereomer is not feasible, as the ion transitions are the same, chromatographic separation of MK-0974 and 1c is warranted. The analytical column was changed from FluoPhase PFP (50 mm × 3 mm, 5 μm) [4] to FluoPhase RP (100 mm × 2.1 mm, 5 μm) to increase resolving power, while keeping the HTLC on-line extraction column and mobile phase compositions unchanged. To accommodate the new column dimension (50 mm versus 100 mm in column length), the flow rate on the analytical column was changed from 0.5 to 0.2 mL/min; and the corresponding time span for each step was modified to ensure enough time for eluting, cleaning and equilibration on the new analytical column. The total run time was changed from 6.5 to 10 min per injection. The modified LC method was applied to both HTLC-A and HTLC-B assays, while the dynamic range was 0.5–500 nM for HTLC-A, and adjusted to 5–5000 nM for HTLC-B once the clinical dose level was defined. Similar to the previously reported [4], the carryover was kept at less than 20% of the lower limit-of-quantification (LLOQ) for both assays.

As the program moved forward, more support from multiple analysts using multiple instruments became critical. On-line extraction instrumentation is not available in every bioanalytical laboratory. To facilitate assay transfer, an off-line SPE assay was developed. Since the on-line extraction column sorbent was reversed-phase C_{18}, the extraction principle was easily transferred by choosing Oasis®HLB μElution SPE plate for sample cleanup. The preparation of sample mix before loading on SPE plate was kept the same as that in on-line extraction, including acidification of the sample to disturb non-specific protein binding. The wash step using 10% acetonitrile further cleaned up the sample matrix without losing the analyte of interest. The elution step using 100% acetonitrile ensured high recovery of the analyte and easy evaporation of the elution solvent before reconstitution. The HPLC condition was modified using isocratic mobile phase on a longer FluoPhase RP (150 mm × 2.1 mm, 5 μm) column to separate the diastereomer from MK-0974 chromatographically without extra time for column equilibration. As a result, the run time was reduced to 5 min per analytical run.

Representative extracted ion chromatograms from human plasma using HTLC-A, HTLC-B and SPE assays are presented in Figs. 2–4, showing a resolution (Rs) of 1.27, 1.29 and 1.18, respectively, which was enough for separation of MK-0974 and its diastereomer (1c), taking advantage that 1c was eluted as a smaller peak in front of MK-0974. Elimination of diastereomer interference was accomplished by using an appropriate non-chiral column within a reasonably short run time.

3.2. Validation of the developed on-line and off-line extraction assays

The on-line HTLC assays (HTLC-A and HTLC-B) and off-line SPE assay were validated to ensure the quality of clinical data. The assay specificity was established by separation of the diastereomer from MK-0974, and the selectivity was demonstrated by using six different lots of human control plasma. The representative chromatograms demonstrated that the assays were specific and selective without interference within the retention time windows of MK-0974 and its IS (Figs. 2–4).

The intra-day variability was evaluated with five different lots of control human plasma spiked with MK-0974 over the calibration

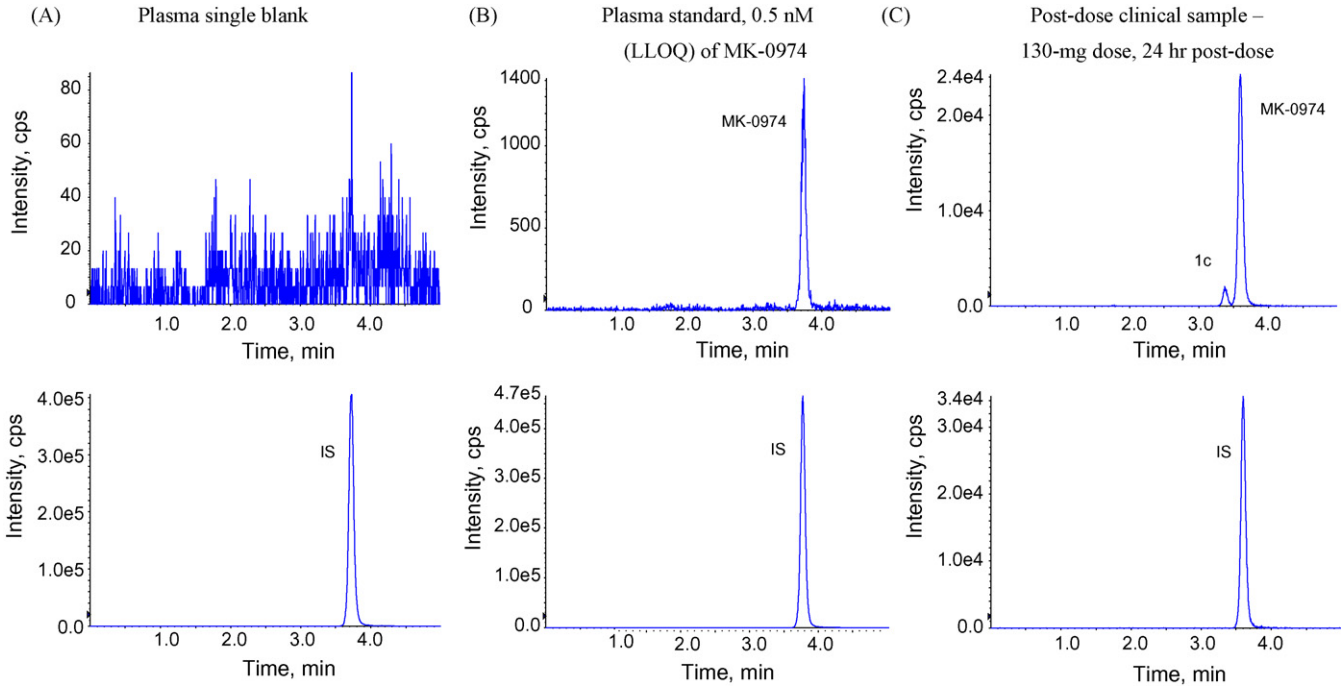

Fig. 2. Representative extracted ion chromatograms from human plasma using Cohesive HTLC on-line extraction assay–HTLC-A: (A) single blank; (B) 0.5 nM standard–lower limit-of-quantification (LLOQ); (C) 24-h post-dose clinical sample following a 130-mg single oral dose of MK-0974 (upper panel: MK-0974; lower panel: IS).

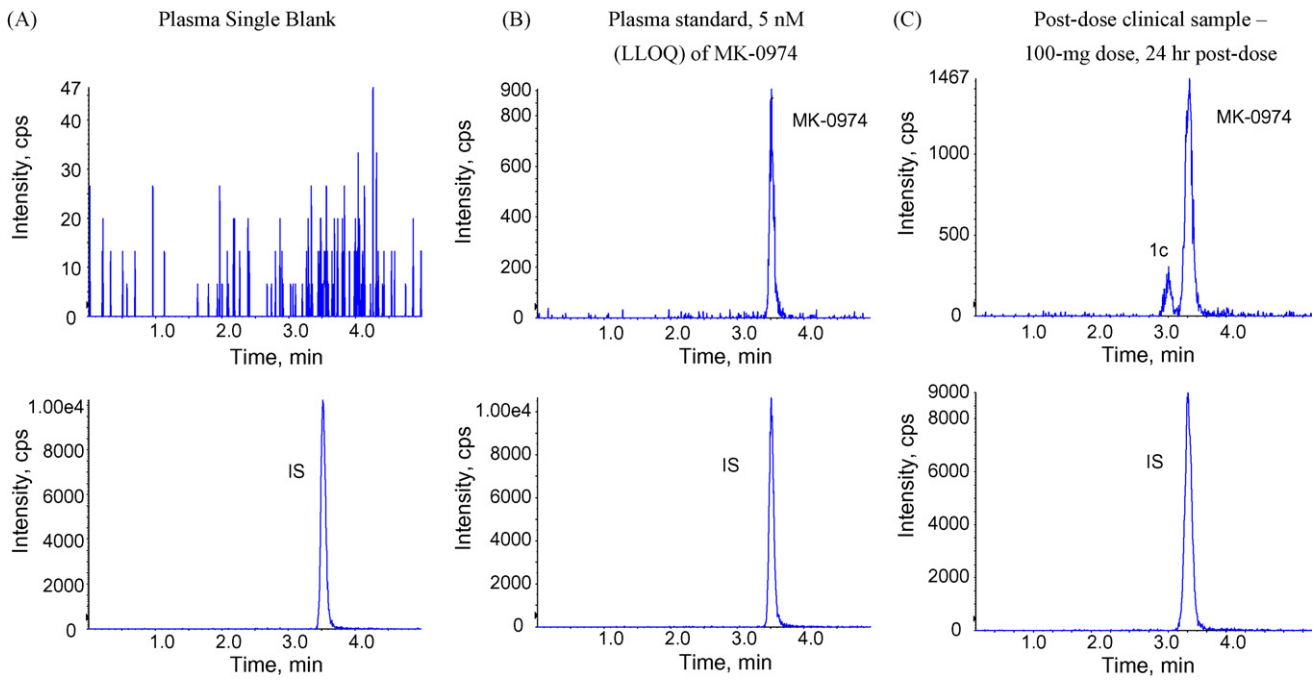

Fig. 3. Representative extracted ion chromatograms from human plasma using Cohesive HTLC on-line extraction assay–HTLC-B: (A) single blank; (B) 5 nM standard–lower limit-of-quantification (LLOQ); (C) 24-h post-dose clinical sample following a 100-mg single oral dose of MK-0974 (upper panel: MK-0974; lower panel: IS).

range of 0.5–500 nM for HTLC-A on an API4000, and 5–5000 nM for HTLC-B and SPE assays on an API3000, with their corresponding LLOQ at 0.5 nM and 5 nM, 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 <3.66%, <6.09 and <5.15% for HTLC-A, HTLC-B and SPE assays, respectively, at each concentration of MK-0974 on the calibration curves. Assay accuracy was found to be 97.97–101.60%, 97.50–101.40% and 98.38–101.53% of nominal MK-0974 concentrations for HTLC-A, HTLC-B and SPE assays, respectively (Table 2).

Quality control (QC) samples containing MK-0974 were prepared at 1.5 (three times the concentration of the LLOQ), 20 and 400 nM for HTLC-A, and 15, 400 and 4000 nM for HTLC-B and SPE assays. In order to demonstrate the ability to dilute samples above the upper limit of the standard curve, 10× HQC samples, 4000 nM in HTLC-A and 40000 nM in HTLC-B and SPE, were prepared and analyzed with 10-fold dilution. The results of the initial analyses ($n = 5$) of these samples are shown in Table 3.

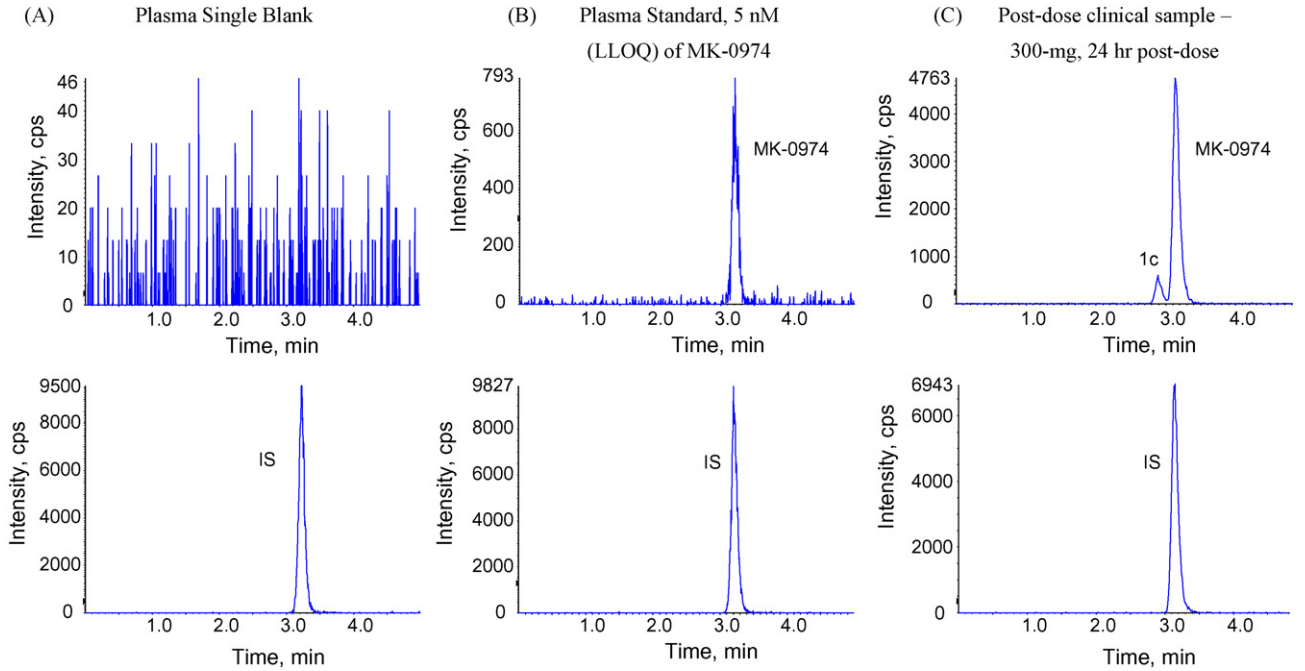

Fig. 4. Representative extracted ion chromatograms from human plasma using off-line SPE extraction assay: (A) single blank; (B) 5 nM standard–LLOQ; (C) 24-h post-dose clinical sample following a 300-mg single oral dose of MK-0974 (upper panel: MK-0974; lower panel: IS).

Table 2
Intra-day validation for the determination of MK-0974 in five lots of control human plasma using HTLC-A, HTLC-B and SPE methods.

On API4000 Nominal conc. (nM)	HTLC-A Accuracy %[a] (%CV)[b]	On API3000 Nominal conc. (nM)	HTLC-B Accuracy %[a] (%CV)[b]	SPE Accuracy %[a] (%CV)[b]
0.50	100.00 (3.66)	5	100.00 (6.09)	100.20 (4.59)
1.00	101.00 (1.13)	10	99.90 (1.80)	99.00 (5.15)
5.00	101.60 (0.90)	20	100.60 (3.13)	101.20 (3.31)
50.00	99.78 (1.32)	200	97.50 (2.18)	98.38 (3.26)
100.00	100.94 (1.06)	800	99.60 (1.48)	98.97 (2.05)
200.00	99.80 (0.96)	2000	101.40 (0.80)	101.53 (0.81)
400.00	99.67 (2.32)	4000	100.80 (0.77)	100.76 (2.43)
500.00	97.97 (0.46)	5000	100.27 (0.82)	99.97 (0.98)

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

Table 3
Initial QC analysis and dilution integrity of human plasma QCs containing MK-0974 determined by HTLC-A, HTLC-B and SPE methods.

Method	Nominal conc. (nM)	Mean conc. (nM) (n = 5)	Accuracy[a] (%)	Precision[b] (%CV)
HTLC-A	1.50	1.51	100.67	2.65
	20	20.53	102.65	1.07
	400	404.09	101.02	1.67
	4000 (10× dilution)	4092.66	102.32	0.95
HTLC-B	15	14.68	97.90	4.25
	400	405.36	101.34	2.10
	4000	3991.08	99.80	2.32
	40000 (10× dilution)	43472.96	108.68	2.67
SPE	15	15.54	103.60	2.19
	400	416.43	104.11	2.16
	4000	4120.44	103.01	2.46
	40000 (10× dilution)	41522.88	103.81	2.31

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

Since HTLC-A inherited the same sample preparation procedures before loading on Cohesive on-line extraction with the same dynamic range, all stability established previously at 1.5, 20 and 400 nM MK-0974 [4] was valid for HTLC-A method. Additional freeze–thaw (after 3 freeze–thaw cycles) and room temperature (at least 4 h at room temperature) stability tests at 4000 nM MK-0974 were conducted to cover the higher dynamic range 5–5000 nM, used in HTLC-B and SPE assays. As injection composition was adjusted for both the HTLC-B and SPE assay, the autosampler stability (cover the time prior to injection that samples are stored in the cooled autosampler) was evaluated at 15, 400 and 4000 nM MK-0974 QC concentrations. Re-injection of five intra-day standard curves after storage at 4 °C for 5 days met the same precision and accuracy criteria as the originally injected samples (data not shown). The long-term stability at −70 °C was established during routine sample analysis. All the experiments were performed as described in Section 2. The results, summarized in Table 4, indicated that there were no stability issues for plasma assays under the tested conditions.

Extraction recovery was evaluated for HTLC-A, HTLC-B and SPE assays, respectively. For the HTLC on-line extraction, recovery 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). MK-0974 extraction recovery was evaluated at 0.5, 50, and 500 nM containing 20 nM IS for HTLC-A on an API4000, and at 5, 200 and 5000 nM for HTLC-B on an API3000. Since the recovery reported here only reflected the extraction of analyte from neat solution, not from plasma, it can only serve as a reference value for the existing extraction method. For the off-line SPE assay, the extraction recovery was determined by comparing five replicates of plasma standards spiked before SPE extraction (pre-spiked) versus standards spiked after extraction of blank plasma (post-spiked) at 5, 200 and 5000 nM MK-0974 levels. As presented in Table 5, the mean recoveries ranged 87.4–96.2% and

Table 4
Stability of MK-0974 QCs in human plasma.

Nominal conc. (nM)	3 F/T[a] (%CV) (%)	RT-4h[b] (%CV) (%)	AS[c] (%CV) (%)		Long-term[d] at −70 °C	
			HTLC-B (48 h)	SPE (20 h)	(%CV) (%)	Duration
1.5	98.9 (3.2)[e]	103.5 (1.6)[e]	NR	NR	107.3 (0.6)	6 months
15	97.7 (2.4)	102.9 (0.7)	100.3 (3.0)	100.5 (3.3)	101.0 (1.5)	13 months
20	99.5 (1.4)[e]	101.2 (2.1)[e]	NR	NR	108.2 (0.4)	6 months
400	98.6 (0.8)[e]	100.8 (2.0)[e]	100.2 (1.6)	101.5 (2.6)	101.9 (2.8)	15 months
4000	101.7 (1.7)	104.0 (3.0)	102.4 (1.7)	102.8 (2.6)	102.7 (2.7)	15 months
40000	ND	ND	ND	ND	99.6 (6.8)	11 months

NR: not relevant, as 1.5 and 20 nM MK-0974 concentrations were only used in the HTLC-A assay; ND: not determined.
[a] Freeze/thaw stability, expressed as [(mean concentration after 3 freeze/thaw cycles)/(initial mean concentration in Table 3) × 100] (%).
[b] Room temperature stability, expressed as [(mean concentration after storage at room temperature for 4 h)/(initial mean concentration) × 100] (%).
[c] Autosampler stability, expressed as [(mean concentration after storage in autosampler for certain time)/(initial mean concentration) × 100] (%).
[d] Long-term stability, expressed as [(mean concentration after storage at −70 °C)/(initial mean concentration) × 100] (%).
[e] Freeze/thaw and room temperature stability at these concentrations was previously established and reported [4].

Table 5
Relative extraction recovery and matrix effects on ionization in human plasma assays, HTLC-A, HTLC-B and SPE.

Method	Analyte	Nominal conc. (nM)	%CV on peak area after extraction	Mean recovery[a] (%)	Mean matrix effect[b] (%)
HTLC-A	MK-0974 (n = 5)	0.50	5.74	88.8	93.3
		50	1.55	96.2	86.9
		500	1.26	90.5	88.1
	IS (n = 15)	20	<2.52[c]	93.5	87.4
HTLC-B	MK-0974 (n = 5)	5	4.25	87.4	119.7
		200	2.00	91.1	119.2
		5000	0.80	93.9	118.5
	IS (n = 15)	80	<3.21[c]	86.2	118.7
SPE	MK-0974 (n = 5)	5	3.73	103.3	89.1
		200	5.06	99.9	100.0
		5000	8.05	105.1	96.3
	IS (n = 15)	80	<9.87[c]	103.2	95.6

Recovery for SPE assay is expressed as [(mean peak area of pre-spiked/mean peak area of post-spiked] × 100] (%).
Matrix effect for HTLC assays is expressed as [(mean peak area of post-spiked/mean peak area of neat solution) × 100] (%).
[a] Recovery for HTLC assays is expressed as [(mean peak area of neat after extraction/mean peak area of neat without extraction column) × 100] (%).
[b] Matrix effect for HTLC assays is expressed as [(mean peak area of MK-0974 in plasma after extraction/mean peak area of neat after extraction) × 100] (%).
[c] The %CV observed for IS under three concentrations of parent MK-0974 (n = 3).

99.9–105.1% for on-line and off-line extraction, respectively. Internal standard recovery was around 94% for on-line extraction and 103% for off-line extraction (Table 5).

Two approaches were utilized to assess matrix enhancement/suppression of ionization. For on-line extraction, the matrix effect was evaluated by comparing the absolute peak area of plasma (at 0.5, 50 and 500 nM for HTLC-A or 5, 200 and 5000 nM for HTLC-B) samples after extraction to that of neat standard after extraction. Strictly speaking, this value reflected the combination of matrix effect and recovery differences between samples and neat solutions. For off-line extraction, the matrix effect was assessed by taking the mean (n = 5) peak area ratio of post-spiked standards (at 5, 200 and 5000 nM) versus that of neat analyte solution. As the result, less than 20% of ion suppression/enhancement was observed for all three assays (Table 5). Referring to the intra-day precision and accuracy obtained from five different lots of plasma (Table 1), the observed absolute matrix effect did not have any significant impact on the assay performance.

3.3. Applications of the validated on-line and off-line extraction assays to clinical study samples and cross-validation

The HTLC-A, HTLC-B and SPE assays have been used to support multiple clinical studies. Representative chromatograms of human clinical samples from subjects dosed with MK-0974 using HTLC-A, HTLC-B and SPE methods are shown in Figs. 2C, 3C and 4C, respectively. The measured MK-0974 concentrations in plasma were within the corresponding dynamic ranges, otherwise, appropriate dilution was performed. The mean QC values calculated from multiple runs during daily analysis are summarized in Table 6. Good precision and accuracy results indicate all three assays were rugged and consistent.

Furthermore, a cross-validation was conducted using clinical post-dose study samples. At each sampling time point, clinical samples from 6 subjects administered 400 mg MK-0974 was pooled and analyzed using on-line HTLC-B and off-line SPE assays, respectively. The results demonstrated that there was no significant difference (<2.6%) between the data generated with on-line and off-line extraction methods (Table 7).

3.4. Pros and cons of HTLC on-line extraction and SPE off-line extraction in the case of MK-0974 analysis

HTLC on-line (HTLC-A and HTLC-B) and SPE off-line extraction methods for the LC–MS/MS analysis of MK-0974 have been developed to eliminate the interference from a diastereomer. All methods fulfilled critical validation criteria satisfactorily, and the cross-validation results showed good comparability between on-line and off-line assays. All methods were successfully applied to the determination of MK-0974 plasma concentrations in clinical pharmacokinetic studies.

Table 6
Mean concentrations of QCs obtained during daily analysis in clinical studies using HTLC-A, HTLC-B and SPE assays.

Method	Nominal conc. (nM)	Mean conc. (nM)	Accuracy[a] (%)	Precision[b] (%CV)
HTLC-A (n = 11)[c,d]	1.50	1.54	102.67	4.55
	20	21.20	106.00	3.11
	400	418.94	104.74	3.50
HTLC-B (n = 27)[c,e]	15	14.96	99.73	3.74
	400	405.15	101.29	4.23
	4000	4130.29	103.26	3.23
SPE (n = 9)[c,f]	15	14.75	98.33	4.95
	400	413.24	103.31	4.41
	4000	3931.43	98.29	3.89

[a] Expressed as [(mean calculated concentration)/(nominal concentration) × 100] (%).
[b] Expressed as coefficient of variation (%CV).
[c] n is the number of runs performed, while in each run, there were typically 2–4 sets of QCs analyzed.
[d] Operated by 2 analysts on 1 Cohesive system, from study 001.
[e] Operated by 2 analysts on 2 Cohesive systems, from study 005.
[f] Operated by 7 analysts on 6 mass spectrometers, from study 017.

Table 7
Cross-comparison of concentration data from clinical samples analyzed using on-line extraction (HTLC-B) and off-line SPE assays.

Time of post-dose (h)	Pooled conc.[a] (nM) from HTLC-B	Pooled conc.[a] (nM) from SPE assay	SPE/HTLC-B[b] (%)
1	781.97	784.00	100.26
2	1543.64	1530.00	99.12
4	2423.03	2360.00	97.40
8	701.70	687.00	97.91
16	132.65	130.00	98.00
24	68.03	67.70	99.51

[a] Pooled samples obtained from 6 subjects at each time point.
[b] Calculated as [concentration observed from SPE/concentration observed from HTLC-B] × 100 (%).

Table 8
Pros and cons of Cohesive HTLC on-line extraction and SPE off-line extraction in the case of MK-0974 analysis.

	HTLC on-line extraction	SPE off-line extraction
Sample off-line cleanup	No need	~1–2 h/96-well plate; extra-man power for sample cleanup
LC–MS/MS run time	10 min/sample; ~16 h/96-well plate	5 min/sample (isocratic); ~8 h/96-well plate
Carryover	Generally <20% of LLOQ in 1000-fold assay dynamic range [4]	None or very minimal in a 1000-fold dynamic range
Assay dynamic range	1000-fold for MK-0974	1000-fold for MK-0974
Instrument cost	Cohesive instrument, including pumps and autosampler, ~$110 K	TomTec ~55 K; HPLC system including autosampler ~50–70 K
Daily extraction cost	~$395/extraction column for ~2000 samples, equivalent to $0.20/sample	~$270/SPE plate for 96 sample; equivalent to $2.81/sample
Assay transfer	Relatively challenging depending on the models of instruments and training of analysts	Relatively easy and fast

Knowing the pros and cons of different methods will allow better decision-making about extraction procedure selection for clinical study support. A comparison of the two approaches, on-line versus off-line extractions, are summarized in Table 8 with regard to man power involved in sample preparation, LC–MS/MS analysis time, carryover, dynamic range, instrument cost, daily extraction cost and the requirements for assay transfer. Along this line, each point is further discussed in detail.

Turbulent-flow liquid chromatography has been shown to eliminate the need for traditional off-line sample preparation as it allows direct injection of plasma or serum samples [6–14]. An important feature and advantage of on-line extraction, compared with off-line SPE, is direct elution of the analyte from the extraction sorbent into the LC system. The extra off-line sample cleanup steps, such as equilibrium of SPE plate, loading, washing, eluting, etc., are eliminated, making on-line extraction more efficient and fully automated. In contrast, SPE off-line extraction required an extra 1–2 h for processing every 96-well plate where some intervention by the operator is needed. It is worth mentioning that, unlike the conventional SPE plate, Waters Oasis®HLB μElution 96-well SPE plate provides high recovery and fast workflow without delay due to plugging of extraction wells during sample loading. However, the time needed for off-line sample preparation, although minimized, could become more significant when multiple plates (e.g. 4 plates/analyst) are handled in the same day.

The LC run time for SPE assay was about half of that for HTLC method because of the special need for diastereomer separation. To obtain enough resolving power, a longer analytical column was used. Subsequently, the time for elution, washing and equilibration of the column on HTLC became a significant factor, while the isocratic SPE method chromatography avoided the time-consuming washing and equilibration steps. With the availability of parallel HTLC systems, these time-consuming steps became less of an issue. The HTLC assay has been successfully transferred to a Cohesive TX2 HTLC 2300 system to perform two analyses in the time taken for one analysis using serial mode (data not shown).

One of the challenges associates with HTLC on-line extraction is carryover. Analysts usually have to spend time developing approaches that minimize carryover; even so, the assay dynamic range sometimes has to be truncated to make carryover insignificant. The relative carryover is often calculated as (analyte peak area in a double blank injected immediately after the upper limit-of-quantification sample/analyte peak area of the lower limit-of-quantification) × 100%. The strategy to minimize carryover in developing a HTLC assay has been reported, with which, the MK-0974 on-line assay was able to provide <20% carryover for the plasma assay that had 1000-fold dynamic range [4]. In comparison, the SPE off-line assay for determination of MK-0974 showed no or very minimal carryover which made developing an assay with larger dynamic range possible. One example is the previously reported off-line SPE MK-0974 chiral assay [5]. Since the concentrations of MK-0974 were much higher than its isomers in the clinical samples, to avoid two-step analysis (i.e. measuring MK-0974 with dilution and others without dilution), the dynamic range of 10,000-fold was validated for MK-0974 with <20% carryover. From this perspective, the off-line SPE assay demonstrates an advantage over the on-line HTLC approach.

Cohesive HTLC on-line extraction is more cost efficient than the automated SPE off-line method. The instrument costs for both methods are comparable because an automated liquid handling system, TomTec, was used to handle SPE extraction in a 96-well format. However, the daily cost of on-line extraction is significantly reduced because of the extended life time of the extraction column, using the mobile phase combination of 15% acetic acid (mobile phase C) and 10% acetonitrile in tetrahydrofuran (mobile phase D) to wash away plasma proteins and lipids [1,15]. The daily extraction cost for SPE plate could potentially be ~14-fold more expensive then using the HTLC extraction column.

As drug development progresses, the clinical studies get larger, and therefore, there is a critical need for assay transfer either to external contract research organizations (CROs) or to internal analytical groups handled by more analysts. Assay transfer is more challenging for on-line extraction assays because of two reasons. First, personnel require more training and instrument familiarization time. Second instrumentation, as Cohesive systems are incorporating pumps from different venders (e.g., Agilent, Shimadzu, Flux pumps, etc.) with different dead volumes. The timing of column switching cannot be directly transferred from one pump system to the other. Modification on each step of the LC method will need time and experienced analysts. In comparison, the SPE assay using conventional HPLC system is easier to handle; and the assay transfer, especially for isocratic conditions, is fast and straightforward.

4. Conclusions

The HTLC on-line extraction and SPE off-line extraction assays have been developed and modified in a timely manner to meet clinical study support needs. The interference caused by the diastereomer of MK-0974 was eliminated to ensure the specificity of MK-0974 determination. Both assays were robust, sensitive, spe-

cific, accurate and reliable. As a result of comparison, the HTLC on-line extraction method appears to be more cost effective and operationally efficient to support clinical studies in the sense of using minimal man power to handle thousands of human samples, while the SPE off-line extraction method enables easy assay transfer among multiple laboratories and analysts and exhibits less carryover. Knowing the pros and cons of different technologies will ensure better decision-making in support of clinical studies.

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