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# A simplified and completely automated workflow for regulated LC–MS/MS bioanalysis using cap-piercing direct sampling and evaporation-free solid phase extraction

Naiyu Zheng[a], Adela Buzescu[a], Stephanie Pasas-Farmer[a,1], Mark E. Arnold[a], Zheng Ouyang[b], Mohammed Jemal[b], Qianping Peng[c], Terry Van Vleet[d], Jianing Zeng[a,*]

[a] Analytical & Bioanalytical Development, Bristol-Myers Squibb Company, Princeton, NJ 08540, United States
[b] Bioanalytical & Discovery Analytical Sciences, Bristol-Myers Squibb Company, Princeton, NJ 08540, United States
[c] Drug Safety Evaluation, Bristol-Myers Squibb Company, New Brunswick, NJ 08903, United States
[d] Drug Safety Evaluation, Bristol-Myers Squibb Company, Mt. Vernon, IN 47620, United States

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ABSTRACT

Automated sample extraction for regulated bioanalysis by liquid chromatography/tandem mass spectrometry (LC–MS/MS) still presents significant challenges. A new sample preparation methodology with a simplified and completely automated workflow was developed to overcome these challenges using cap piercing for direct biofluid transfer and evaporation-free solid phase extraction (SPE). Using pierceable cap sample tubes, a robotic liquid handler was able to sample without uncapping or recapping during sample preparation. Evaporation for SPE was eliminated by using a mobile phase-compatible elution solvent followed by sample dilution prior to LC–MS/MS analysis. Presented here are three LC–MS/MS assays validated using this methodology to support three CNS drug development programs: (1) BMS-763534 and its metabolite, BMS-790318, in dog plasma; (2) BMS-694153 in monkey plasma; and (3) Pexacerfont (BMS-562086) and two metabolites, BMS-749241 and DPH-123554, in human plasma. These assays were linear from 1.00 to 1000 or 2.00 to 2000 ng/mL for each analyte with excellent assay accuracy, precision and reproducibility. These assays met acceptance criteria for regulated bioanalysis and have been successfully applied to drug development study samples. The methodology described here successfully eliminated all manual intervention steps achieving fully automated sample preparation without compromising assay performance. Importantly, this methodology eliminates the potential exposure of the bioanalyst to any infectious biofluids during sample preparation.



## 1. Introduction

Throughput for liquid chromatography/tandem mass spectrometry (LC–MS/MS) bioanalytical methods is often limited by the complexity and time involved with sample preparation [1–5]. Due to labor intensive and time consuming steps associated with manual sample preparation, parallel sample processing in a 96-well format (or 96-tube cluster format) using a robotic liquid handler has been widely adopted for routine bioanalysis in drug discovery and development [6–16]. During clinical drug studies, large numbers of samples are usually collected from multiple sites, where it is not always practical to collect the samples in a 96-well plate due to the concerns of operational errors, sample contamination or sample traceability in each sample handling step. Therefore, collection and storage of the biofluids in capped polypropylene tubes with proper labeling is still preferred in regulated bioanalysis [13,17]. Consequently, regardless of which sample extraction method is used (e.g., protein precipitation, liquid–liquid extraction or solid phase extraction), all biofluid subsampling to a 96-well plate is accomplished by manually uncapping and recapping of the sample tubes (Fig. 1A) [13,17,18], which has become the bottleneck for automated sample extraction. While universal precautions are observed, the potential health risk still exists for accidental exposure to the infectious biofluids (such as samples collected from HCV or HIV patients) or at risk for potential occupational injuries due to repetitive movement of their hands to uncap or recap the samples tubes [19].

Previously, using pierceable caps for sample collection tubes was introduced to eliminate the need for uncapping and recapping during sample analysis (as shown in Fig. 1B) [19]. In support

* Corresponding author. Tel.: +1 609 252 5669; fax: +1 609 252 3845.
*E-mail address:* jianing.zeng@bms.com (J. Zeng).
[1] Current address: WuXi AppTec, Inc., 4751 League Island Blvd, Philadelphia, PA 19112, United States.

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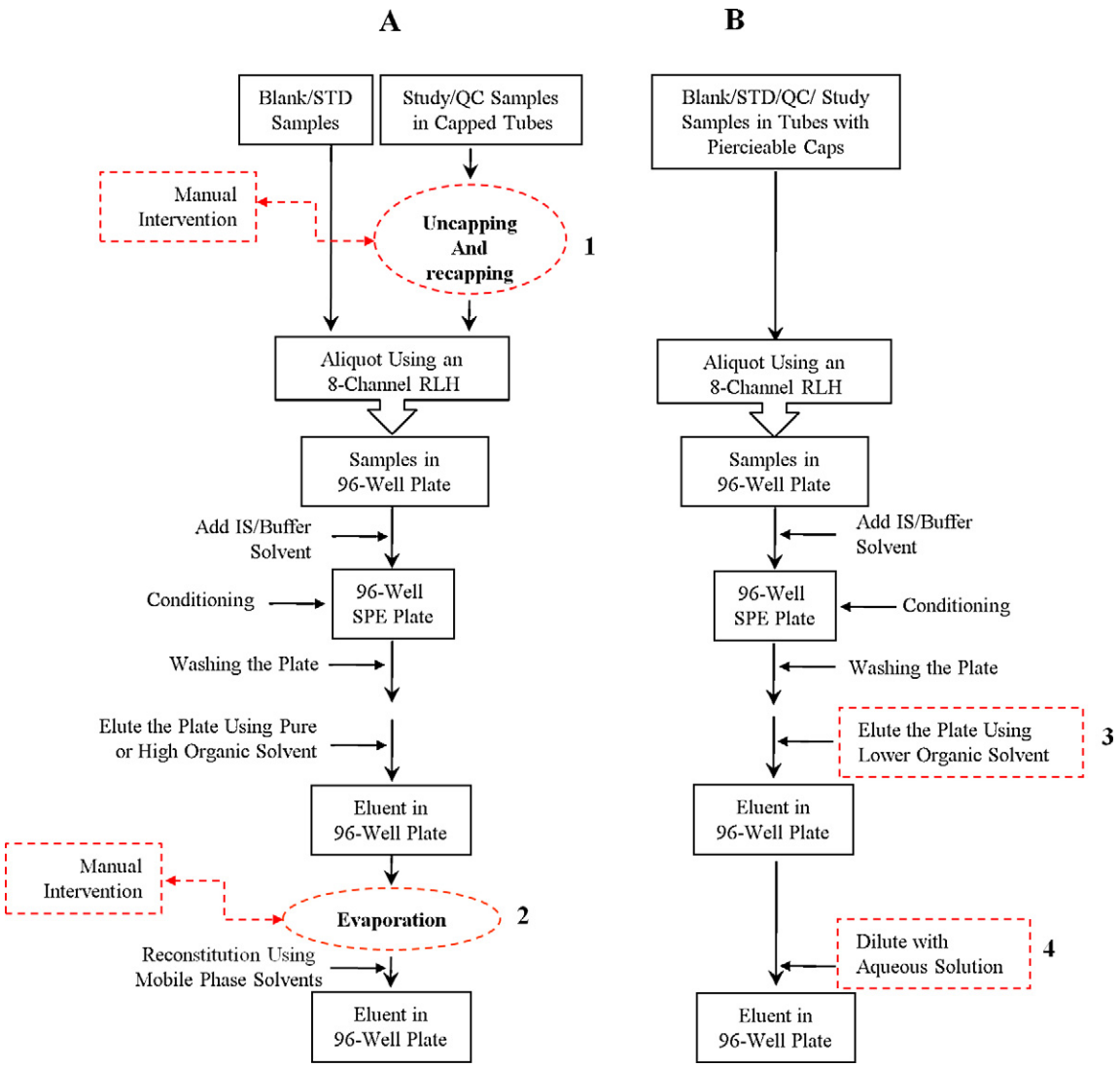


**Fig. 1.** Illustration of two SPE sample extraction processes for bioanalysis using LC–MS/MS: (A) conventional SPE sample extraction with a robotic liquid handler (RLH), in which steps 1 and 2 required manual interventions; (B) direct biofluid transfer with evaporation-free SPE sample extraction, in which no manual interventions are required for steps 1 and 2. The evaporation step was eliminated by modification of elution solvent followed by dilution (steps 3 and 4).

of investigational new drug (IND)-enabling toxicokinetic studies, a typical plasma sample volume collected from rats or mice may be as low as 100–200 μL, and a typical plasma volume of 25–50 μL is used for the assays. The sample tubes designed earlier [19], with an internal diameter as large as 16 mm and the tube dimensions of 56 mm × 16 mm were not suitable for collection of smaller sample volumes (e.g., 100–200 μL) placed in these tubes since it was difficult to accurately aliquot such small volumes using a robotic liquid handler. In this manuscript, a newly designed sample tube with a conical shape at the bottom is introduced to enable a robotic liquid handler to achieve accurate sample aliquoting applicable to regulated bioanalysis from typical animal studies (preclinical) to human studies (clinical) (Fig. 2). The new sample pipetting procedure based on the pierceable caps is generic and suitable for sample volume transfers as low as 25–50 μL from the tubes with sample volumes ranging from 100 μL to 5 mL.

For automated sample extraction, solid phase extraction (SPE) in a 96-well format has been widely used in drug discovery and development [1–3,5]. Traditionally, sample evaporation and reconstitution after sample elution from the SPE plate is usually required before injecting into LC–MS/MS analysis (Fig. 1A). However, in most of the cases, sample evaporation step still requires manual operation, which has become another challenge in automated sample analysis. The purpose of sample evaporation is mainly to improve the analyte detection limit by concentrating the samples from a larger volume to a smaller volume, or to redissolve the dried residue in a reconstitution solution compatible with the HPLC mobile phase. Recently, with the emergence of high sensitive LC–MS/MS instrumentation, it is possible to quantify most analytes in biological samples at a very low concentration without the need to concentrate the analytes. As a result, it is possible to use an HPLC-friendly solvent for SPE elution aimed at elimination of the sample evaporation rather than achieving a higher extraction recovery. In most cases, the lower extraction recovery due to use of a weaker elution solvent will be compensated for by internal standards with no impact on the assay performance, especially when stable isotope-labeled internal standards are used for the assay. Previously, we introduced an evaporation-free solid phase extraction (SPE) methodology aimed at overcoming the bottleneck in automating solid phase extraction at the 54th American Society

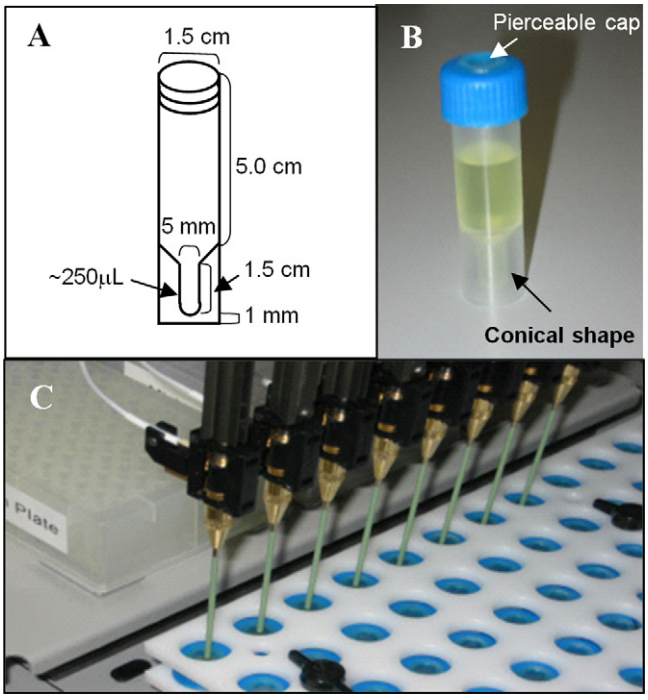


**Fig. 2.** (A) Size and dimension of a plasma sample tube; (B) a plasma sample tube with a pierceable cap; (C) aliquoting of plasma samples by TECAN Genesis fixed tips via cap piercing for subsequent transfer to a 96-well plate.

of Mass Spectrometry Conference (Seattle, WA, USA, 2006), and the preliminary results illustrated the feasibility of eliminating sample evaporation and reconstitution steps using appropriate elution solvents in combination with a final dilution, prior to LC–MS/MS analysis (Fig. 1B). Recently, several reports have shown that evaporation-free SPE methodology resulted in excellent assay performance [20–22] and offered several advantages as compared with the traditional SPE [20,21], such as reducing the cost of equipment and solvent, minimizing sample contamination or analyte conversion, and increasing assay robustness. In this paper, we present the details of our evaporation-free SPE methodology and illustrate its utility in conjunction with a custom-made sample tube design that included a pierceable cap enabling a direct automated sample transfer, which successfully eliminates the initial manual intervention step in sample preparation, and achieves fully automated sample extraction (Fig. 1B).

BMS-763534 (I) [23] and pexacerfont (BMS-562086, IV) [24] are Corticotropin Releasing Factor-1 (CRF1) receptor antagonists that were in development for the treatment of depression and anxiety. BMS-694153 (III) [25] is a calcitonin gene-related peptide (CGRP) receptor antagonist that was in development for the treatment of migraine headaches. To support IND-enabling toxicokinetic studies on BMS-763534 and BMS-694153, and the first-in-human (FIH) study for pexacerfont, three robust and accurate assays have been developed using automated cap piercing for direct sample transfer and evaporation-free solid phase extraction (the structures of the analytes and their internal standards are shown in Fig. 3, the assays are shown in Table 1): (1) BMS-763534 (I) and its metabolite, BMS-790318 (II) in dog plasma (GLP study); (2) BMS-694153 (III) in monkey plasma (GLP study); and (3) Pexacerfont (IV) and its metabolites, BMS-749241 (V) and DPH-123554 (VI), in human plasma (clinical study). To the best our knowledge, this work represents the first reporting of an automated sample processing using generic cap piercing for direct plasma sampling and evaporation-free solid phase extraction for regulated bioanalysis.

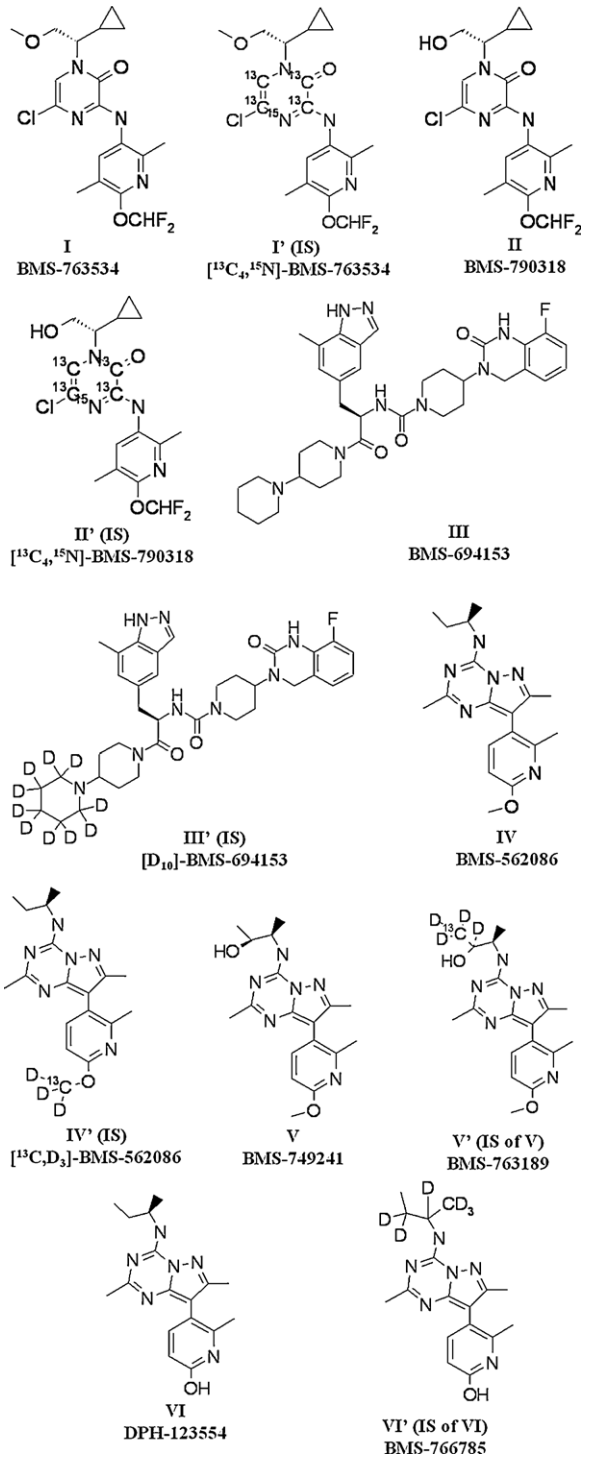


**Fig. 3.** Chemical structures of BMS-763534 (I), BMS-790318 (II), BMS-694153 (III), pexacerfont (BMS-562086, IV), BMS-749241 (V), DPH-123554 (VI) and their internal standards used for the assays: [$^{13}C_4{}^{15}N$]-BMS-763534 (I′), [$^{13}C_4$,$^{15}N$]-BMS-790318 (II′), [$D_{10}$]-BMS-694153 (III′), [$^{13}C$,$D_3$]-BMS-562086 (IV′), BMS-763189 (V′) and BMS-766785 (VI′).

## 2. Experimental

### 2.1. Materials and apparatus

The 96-SPE plates used were Strata-X (10 mg) plate from Phenomenex (Torrance, CA), Isolute-96 C2 (EC, 25 mg) and Evolute™ ABN (10 mg) plates from Biotage, LLC (Charlotte, NC). Plasma

**Table 1**
Three representative assays validated using direct sample transfer and evaporation-free SPE method: curve ranges, assay recoveries and matrix effects.

| Assays | Analytes | SRM transitions | Curve range (ng/mL) | Extraction recovery (%) | Matrix effect | Normalized matrix effect | Mean $R^2$[a] |
|---|---|---|---|---|---|---|---|
| (1) Dog plasma | I | 415 → 249 | 2.00–1000 | 44 | 0.88 | 0.99 | 0.9991 |
| | I′ (IS) | 420 → 254 | | | 0.89 | | |
| | II | 401 → 249 | 2.00–1000 | 53 | 0.88 | 1.00 | 0.9989 |
| | II′ (IS) | 406 → 254 | | | 0.88 | | |
| (2) Monkey plasma | III | 645 → 169 | 1.00–1000 | 58 | 0.97 | 0.94 | 0.9991 |
| | III′ (IS) | 655 → 179 | | | 1.03 | | |
| (3) Human plasma | IV | 341 → 285 | 1.00–1000 | 55 | 1.17 | 0.98 | 0.9991 |
| | IV′ (IS) | 345 → 289 | | | 1.19 | | |
| | V | 357 → 243 | 1.00–1000 | 69 | 1.09 | 1.02 | 0.9998 |
| | V′ (IS) | 362 → 243 | | | 1.07 | | |
| | VI | 327 → 271 | 1.00–1000 | 70 | 1.22 | 1.01 | 0.9996 |
| | VI′ (IS) | 333 → 272 | | | 1.21 | | |

[a] Regression model used: quadratic with weighting factor of 1/*x*.

sample collection tubes were natural polypropylene 5 mL custom-made sample tubes with penetrex caps (VWR catalog number: TX195050BMS, as shown in Fig. 2, hereafter referred to as BMS custom tubes). These tubes were manufactured by Andwin Scientific (Woodland Hills, CA). The reference standards of I–VI and their stable isotope labeled-internal standards, [$^{13}C_4{}^{15}N$]-BMS-763534 (I′), [$^{13}C_4$,$^{15}N$]-BMS-790318 (II′), [$D_{10}$]-BMS-694153 (III′), [$^{13}C$,$D_3$]-BMS-562086 (IV′), BMS-763189 (V′, IS of V, racemic mixture) and BMS-766785 (VI′, IS of VI, racemic mixture), were obtained from Bristol-Myers Squibb (BMS) Research & Development (Princeton, NJ). The structures of the analytes (I–VI) and their internal standards (I′–VI′) are shown in Fig. 3. Control dog, monkey and human EDTA plasma were purchased from Bioreclamation Inc. (Hicksville, NY). Ammonium acetate, ammonium hydroxide and formic acid were purchased from Sigma–Aldrich (St. Louis, MO). Sodium carbonate was purchased from J.T. Baker (Phillipsburg, NJ). HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ). De-ionized water was prepared from an in-house Barnstead Nanopure Diamond system (Dubuque, IA).

### 2.2. TECAN robotic liquid handler

TECAN Genesis 150 robotic liquid handler equipped with an 8-channel liquid handler (LiHa, fixed tip format) arm, TE-VACS vacuum unit and a robotic arm (ROMA) from TECAN US (Durham, NC) was used for sample pipetting and extraction. The system was controlled by Gemini® software. The accuracy and precision of the liquid transfer were verified gravimetrically with a balance on the TECAN work surface. Six replicates of liquid transfers for each tip were performed. A special 96-position sample rack (16 × 6) was custom-made in house by BMS to hold the capped plasma sample tubes [19]. The sample rack had a removable 96-hole cover to hold the capped plasma sample tubes to prevent the entire rack from moving when the fixed tips retracted. The TECAN tips were washed during sample preparation using acetonitrile/water (50/50) with 1% formic acid (for Assay 1) or 40% methanol containing 0.1% formic acid (for Assays 2 and 3). Fig. 4 shows all the labware used for the sample extraction on a TECAN robotic liquid handler.

### 2.3. Evaporation-free solid phase extraction

All plasma samples (blank, calibration standard, quality control and study samples) in BMS custom tubes were pipetted into a 96-well collection plate, followed by adding the internal standard working solution and 0.01 M $Na_2CO_3$ to each well, and mixed well. The 96-well SPE plate [Strata-X plate (10 mg), Isolute-96™ C2 (EC, 25 mg) or Evolute™ ABN plate] was pre-conditioned with methanol followed by 0.01 M $Na_2CO_3$ buffer solution before applying the plasma samples described above. The plate was washed with water and 15% methanol in water, and then eluted with elution solvent. All SPE pre-conditioning, washing and elution steps were performed automatically using on deck TE-VACS vacuum system with parameters that were already included in the TECAN program. The eluent was diluted with water or 20 mM of $NH_4OAc$. An aliquot of 10–25 μL of the sample was injected. The general sample extraction procedure is shown in Fig. 1B and Table 2.

### 2.4. HPLC–MS/MS methods

An LC system consisting of two LC-AD VP binary pumps and a SIL-HT autosampler from Shimadzu Scientific Instrument, Inc. (Columbia, MD), and a TSQ Quantum Ultra™ mass spectrometer with LCQuan 2.5 software from Thermo-Fisher (San Jose, CA) were used for Assay 1. For Assays 2 and 3, an LC system consisting of two LC-AD VP binary pumps and a LEAP HTC-PAL autosampler from Leap Technologies (Carrboro, NC), and an API 4000 mass spectrometer equipped with a TurboIonSpray™ (TIS) source and Analyst Software (v.1.3.1) from AB Sciex (Foster City, CA) were used.

For Assay 1, mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile/water (95/5, v/v). Autosampler wash solution was 0.1% formic acid in acetonitrile/water (75/25, v/v). Atlantics dC18 (50 mm × 2 mm, 5 μm) from Waters (Milford, MA) was used for chromatographic separation with an isocratic elution with B% at 70% for 3.1 min, then increased %B from 70% to 95% in 0.1 min, held for 1.0 min, then decreased %B from 95% to 70% in 0.01 min, held for 1.29 min, stopping the run at 5.5 min. The flow rate was 0.3 mL/min and the column was maintained under ambient conditions. The TSQ Quantum Ultra™ mass spectrometer, equipped with electrospray ionization (ESI) source, was operated in a positive ionization mode. The SRM transitions

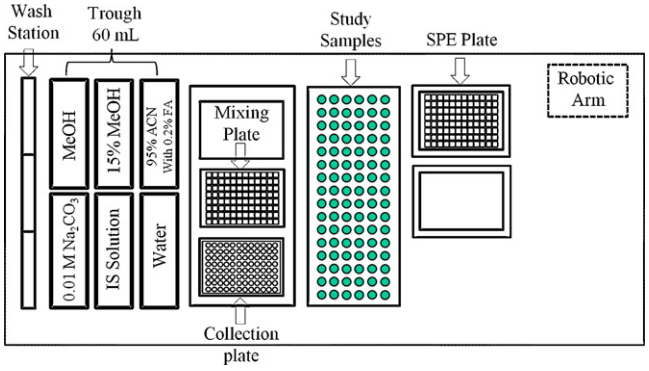


**Fig. 4.** TECAN Genesis® 150 workstation robotic liquid handler deck configuration.

**Table 2**
Generic methodology used for evaporation-free solid phase extraction in three representative assays.

| | Assays | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Plasma (volume, μL) | Dog (50) | Monkey (100) | Human (100) |
| IS solvent (volume, μL) | 30% MeOH/water (50) | 30% MeOH/water (50) | 30% MeOH/water (50) |
| Buffer (volume, μL) | 0.01 M $Na_2CO_3$ (300) | 0.01 M $Na_2CO_3$ (300) | 0.01 M $Na_2CO_3$ (300) |
| 96-Well SPE plate | Strata-X (10 mg) | C2 (25 mg) | Evolute (10 mg) |
| **Extraction** | | | |
| Conditioning 1 (volume, μL) | MeOH (250) | MeOH (250) | MeOH (400) |
| Conditioning 2 (volume, μL) | 0.01 M $Na_2CO_3$ (250) | 0.01 M $Na_2CO_3$ (250) | 0.01 M $Na_2CO_3$ (300) |
| Wash 1 (volume, μL) | Water (300) | Water (300) | Water (300) |
| Wash 2 (volume, μL) | 15% MeOH (200) | 15% MeOH (200) | 15% MeOH (200) |
| Elution solvent (volume, μL) | 0.2% FA in 95% ACN (175) | 20 mM $NH_4OAc$ + 0.2% FA in 65% ACN (150) | 0.2% FA in 95% ACN (250) |
| Dilution solvent (volume, μL) | Water (75) | Water (150) | 20 mm $NH_4OAc$ (250) |
| Final injection sample (volume, μL) | 0.14% FA in 67% ACN (250) | 10 mM $NH_4OAc$ + 0.1% FA in 32.5% ACN (300) | 10 mM $NH_4OAc$ + 0.1% FA in 47.5% ACN (500) |
| Injection volume (μL) | 25 | 10 | 10 |
| Mobile phases at time 0 | 0.1% FA in 66.5% ACN | 10 mM $NH_4OAc$ + 0.1% FA in 34.7% ACN | 10 mM $NH_4OAc$ + 0.1% FA in 44.6% ACN |

used are listed in Table 1. Nitrogen was used as both the sheath and auxiliary gas, and optimized to 49 and 24 psi, respectively. Argon collision gas was used and set to 1.5 mTorr. The electrospray voltage was set at 3000 V, the vaporizer temperature was at 250 °C and the heated capillary temperature was at 275 °C. The MS parameters were optimized with a tube lens offset of 69 and quad MS/MS bias of −1.7 for both analytes.

For Assay 2, mobile phase A was 10 mM ammonium acetate and 0.1% formic acid in acetonitrile/water (5/95, v/v). Mobile phase B was 10 mM ammonium acetate and 0.1% formic acid in acetonitrile/water (95/5, v/v). Autosampler wash solution A was 30% acetonitrile in water containing 0.1% formic acid. Autosampler wash solution B was methanol/isopropyl alcohol/acetone/water (1/1/1/1) containing 0.1% formic acid. A Synergi Polar-RP analytical column (50 mm × 2 mm id, 4 μm) with a SecurityGuard™C18 guard column (4 mm × 3.0 mm ID) from Phenomenex (Torrance, CA) was used for chromatographic separation with an isocratic elution consisting of 33% of mobile phase B. The total run time was 3.5 min. The flow rate was 0.2 mL/min and the column was maintained at ambient temperature. The API 4000 mass spectrometer with a TIS source was operated in the positive ionization mode. The MRM transitions used are listed in Table 1. Nitrogen was used as both the curtain and collision gas, and optimized to 14 and 3 psi, respectively. Ion source gas 1 and gas 2 were set to 40 psi. The TIS voltage was set at 5000 V; the turbo probe temperature (TEM) at 450 °C. Optimal dwell time was 200 ms. The Declustering Potential (DP) was 101. Entrance Potential (EP), the Collision Energy (CE) and Collision Cell Exit Potential (CXP) were 10, 35 and 12, respectively.

For Assay 3, the mobile phases, autosampler wash solutions and HPLC column used were the same as those for Assay 2 as described above; however, the chromatographic separation was performed with a gradient elution. The gradient started with 44% B, stayed for 0.4 min, then increased %B from 44% to 65% in 0.9 min, held for 1.5 min, then increased %B from 65% to 95% in 0.1 min, held for 0.7 min, decreased %B from 95% to 44% in 0.1 min, held for 1.8 min, and stopped the run at 5.5 min. The flow rate was 0.25 mL/min and the column temperature was maintained at ambient condition. The API 4000 mass spectrometer with a TIS source was operated in the positive ionization mode. The MRM transitions used are listed in Table 1. Nitrogen was used as both the curtain and collision gas, and optimized to 10 and 6 psi, respectively. Ion source gas 1 and gas 2 were set to 50 and 40 psi. The TIS voltage was set at 5000 V. TEM was at 400 °C. Optimal dwell time was 200 ms. The DP was 53.2 V, 57.0 V and 43.5 V for IV, V and VI, respectively. EP was 7.0, 7.1 and 8.8 for IV, V and VI, respectively. The CE was 35.0 V, 38.0 V and 31.0 V for IV, V and VI, respectively. CXP was 16.0 V, 13.2 V and 15.0 V for IV, V and VI, respectively.

### 2.5. Method validation

The three assays described were validated according to the FDA Guidance for Bioanalytical Method Validation [26]. All three assays have been fully validated with extensive testing related to analyte stability, which will not be discussed in this manuscript since the focus of this manuscript is the sample extraction strategy and assay performance. The experiment details on STD and QC preparation, recovery and matrix effect evaluation, as well as carryover evaluation for robotic liquid handler can be found in the following sections.

#### 2.5.1. Calibration standard (STD), quality control (QC) and internal standard (IS) preparation

Stock solutions (1.00 mg/mL) for analytes or internal standards were prepared in acetonitrile (for I, II, I′, II′) or methanol (for III–VI and III′–VI′). STD and QC stock solutions were prepared from a separate weighing of the reference material for each analyte. Ten concentration levels of the STD at 2.00, 4.00, 10.0, 25.0, 50.0, 100, 250, 500, 750 and 1000 ng/mL for each analyte (for Assay 1), or 1.00, 2.00, 5.00, 10.0, 50.0, 100, 250, 750 and 1000 ng/mL for each analyte (for Assays 2 and 3) were prepared by spiking the stock solutions into control dog, monkey or human EDTA plasma. Six QC levels, 2.00, 6.00, 50.0, 400, 800 and 5000 ng/mL for each analyte (for Assay 1), or 1.00, 3.00, 35.0, 400, 800 and 5000 for each analyte (for Assays 2 and 3) were prepared in control dog, monkey and human EDTA plasma. The single or combined internal standard working solutions in methanol/water (30/70, v/v) were prepared at 100/100 ng/mL for I′/II′ (Assay 1), 100 ng/mL for III′ (Assay 2) and 25/100/100 ng/mL for IV′/V′/VI′, respectively.

#### 2.5.2. Carryover of the robotic liquid handler

Carryover of the liquid handler with fixed tips was evaluated by using all 8 tips from the liquid handler to transfer a High QC sample followed by washing and then transferring a double blank matrix sample. All the double blank matrix samples were injected first followed by the High QC samples. All transfers for each tip were repeated in three replicates. The carryover of the liquid handler was calculated as the percent response in the blank compared to the response in the High QC sample.

*2.5.3. Extraction recovery and matrix effect*

The extraction recovery of each analyte from the plasma matrix for each assay was determined at Low QC (LQC) and High QC (HQC) by comparing the response ratios in plasma matrix samples spiked with the analyte(s) prior to extraction with those spiked post-extraction. The matrix effect was determined at the concentrations of LQC and HQC by dividing the analyte response (peak area) in plasma matrix spiked post-extraction with analyte(s) by the analyte response of those spiked in reconstitution solution. The matrix effects of the ISs were determined similarly at each concentration used.

*2.5.4. Intra- and inter-assay precision and accuracy*

For each assay, intra- and inter-assay precision and accuracy were assessed by analyzing calibration curve standards in duplicate and quality control samples in six replicates in three validation runs. The accuracy was expressed by mean observed concentration)/(nominal concentration) × 100%. The precision was expressed as percentage relative standard deviation (%Dev).

## 3. Results and discussion

*3.1. Automated cap-piercing for direct biofluid transfer*

The use of the pierceable caps allowed the TECAN robotic liquid handler to pierce through the caps for direct biofluid transfer. The caps used for sample storage should be leak-proof and maintain their leak-proof integrity after piercing, even after long-term storage and/or undergoing several freezing and thawing cycles. The materials used for the manufacturing of the pierceable caps described here were demonstrated to have a good re-sealability after multiple piercing [19]. To facilitate the automation, all study samples were collected in BMS custom tubes (VWR catalog number: TX195050BMS) (Fig. 2) shipped on dry ice to the bioanalytical lab and stored at −20 °C before analysis. All QCs were pre-prepared in the same tubes and caps, and stored at −20 °C. The calibration standard samples were freshly prepared and transferred in the same tubes with caps on the day of analysis. Before sample analysis, the study samples and QC samples were thawed on benchtop, and then placed with the standard curve samples in a 16 × 6 sample rack (Fig. 2C) following the order specified in the sample list generated by Watson® Laboratory Information Management System (LIMS). After brief vortex mixing, the samples in the sample rack were directly transferred using fixed tips on the TECAN Genesis workstation that pierced each cap during sampling.

The accuracy and precision of the volumes transferred by the TECAN Genesis workstation were verified before using for regulated bioanalysis, and based on an internal SOP, the percent deviation of the average liquid transfer should be within ±5.0% of the theoretical value. As shown in Table 3, the TECAN Genesis workstation used in this study demonstrated excellent pipetting accuracies (%Dev ≤ ±2.9% for 50 μL; 1.4% for 900 μL) and precisions (%CV ≤ 1.6% for 50 μL; 1.4% for 900 μL) for all eight pipettor channels.

The total carryover from the robotic liquid handler was evaluated for each analyte. By washing the TECAN tips with wash solutions after each sample transfer, no carryover issue was observed since the total carryover from TECAN liquid handler was under 0.020% for all analytes, which was less than 20.0% of the LLOQ. Since the carryover issue is compound dependent, for certain compounds, aggressive wash (larger volumes or longer wash time) may be necessary.

As discussed above, the one-size-fix-all design of the sample tube had a conical shape at the bottom, which could be used to collect samples from small volumes (100–200) up to 5 mL. When the sample volume was low, the sample was sitting mainly in the bottom portion of the tube; so the TECAN program setting for the area of the sample tube should be based on the area of the bottom part of the tube (∼38.5 $mm^2$), not the area of the upper part of the tube (∼153.9 $mm^2$).

*3.2. Evaporation-free solid phase extraction of plasma samples*

SPE methods usually require sample elution using a large volume of a pure or high concentration of organic solvent to maximize extraction recovery. After evaporation, the eluted sample is reconstituted to the same composition as the LC mobile phases, which usually contains a low percentage of organic solvent. To reduce the gap in the percentage of organic solvents between the elution solvents and HPLC injection solvents, careful selection of the SPE plate and HPLC column is needed to allow the use of a lower organic solvent as the elution solvent and a higher organic solvent as the injection solvent. HPLC columns that have good retention for the polar analytes should be considered first since a higher percentage organic solvent (or stronger organic solvent) can be used as the HPLC elution solvent. Consequently, the solvent percentage difference between the SPE elution and sample reconstitution is smaller; therefore, the constituent of injected sample can match the starting HPLC mobile phases without extensive dilution.

The evaporation-free SPE methodology developed is generic and can be used for multiple assays with minor changes as needed. TECAN labware configuration for all three assays can be found in Fig. 4, and the detailed solutions and SPE plates used can be found in Table 2. For method development, usually HPLC mobile phase B can be used as the initial elution solution for SPE, and then diluted with appropriate volume of aqueous solution after elution. The content of organic solvent and mobile phase additives can be slightly adjusted based on the need of analytes. These approaches have successfully led to the development of many evaporation-free SPE methods in our laboratory including the three methods discussed here.

For Assay 1, the analytes were fairly-well retained on an Atlantics dC18 HPLC column, so that an isocratic elution with as high as 70% of the mobile phase B was possible, which represented a 66.5% of acetonitrile and 0.1% formic acid. To maximize the extraction recovery for both analytes with different polarities, the Strata-X SPE was used since it is known to contain a reversed-phase functionalized polymeric sorbent that gives a strong retention of neutral, acidic, or basic compounds under aggressive, high organic wash conditions. The SPE plate was eluted with 175 μL of 95% ACN containing 0.2% formic acid, then diluted with 75 μL of water and mixed. The final injection solution contained approximately 66.5% acetonitrile with 0.14% formic acid, which was close to the mobile phase needed for LC–MS/MS analysis (66.5% acetonitrile with 0.1% formic acid). Therefore, the diluted sample could be directly injected into LC–MS/MS without the need for evaporation and reconstitution.

For Assay 2, a Synergi Polar-RP HPLC column was used under isocratic elution consisting of 33% Mobile Phase B, which represented 34.7% acetonitrile and 0.1% formic acid. Thus, the HPLC elution solvent contained a low percentage of organic solvent. A low-retention SPE plate, C2 was used to eliminate the need for using a high percentage organic elution solvent. Unlike most traditional SPE methods where 100% organic solvent was used, the C2 SPE plate was eluted with 150 μL of 65% acetonitrile containing 20 mM ammonium acetate and 0.2% formic acid, and then diluted with 150 μL of water. The final injection solution contained approximately 32.5% acetonitrile with 10 mM $NH_4OAc$ and 0.1% formic acid, which was close to the mobile phase used for the LC–MS analysis (34.7% acetonitrile with 10 mM $NH_4OAc$ and 0.1% formic acid).

**Table 3**
TECAN performance verification: liquid transfer accuracy and precision at 50 and 900 μL for each fixed tip ($n = 6$).

| Tip number | 50 μL ($n = 6$) | | | 900 μL ($n = 6$) | | |
|---|---|---|---|---|---|---|
| | Mean weight (mg) | Mean %Dev | %CV | Mean weight (mg) | Mean %Dev | %CV |
| 1 | 50.99 | 2.0 | 0.6 | 910.6 | 1.2 | 0.5 |
| 2 | 51.44 | 2.9 | 0.8 | 905.1 | 0.6 | 1.0 |
| 3 | 50.00 | 0.0 | 0.5 | 910.7 | 1.2 | 1.4 |
| 4 | 50.00 | 0.0 | 0.5 | 909.5 | 1.1 | 0.5 |
| 5 | 50.14 | 0.3 | 1.6 | 911.0 | 1.2 | 0.5 |
| 6 | 50.62 | 1.2 | 0.8 | 910.6 | 1.2 | 0.5 |
| 7 | 50.82 | 1.6 | 0.5 | 911.2 | 1.2 | 0.5 |
| 8 | 50.65 | 1.3 | 0.3 | 912.3 | 1.4 | 0.6 |

Similar to Assay 2, a Synergi Polar-RP HPLC column was used for Assay 3. The initial mobile phase composition (at time 0) contained 44.6% of acetonitrile, 10 mM $NH_4OAc$ and 0.1% formic acid. An EVOLUTE® ABN SPE plate was used to achieve consistent recovery for each compound in spite of their different polarity. EVOLUTE® ABN SPE plate is a water-wettable polymer-based sorbent that can be used to extract acidic, basic and neutral analytes from biological fluids and other aqueous matrices. Each sample in the extraction plate was eluted with 250 μL of 95% ACN containing 0.2% formic acid, and then diluted with 250 μL of 20 mM of $NH_4OAc$. The final injection solution contained approximately 47.5% acetonitrile with 10 mM of $NH_4OAc$ and 0.1% formic acid, which was close to the mobile phase needed for LC–MS/MS analysis (44.6% acetonitrile with 10 mM of $NH_4OAc$ and 0.1% formic acid). Again, the diluted sample could be directly injected into the LC–MS/MS without the need for evaporation and reconstitution.

In the methods presented here, all steps were performed automatically except for vortexing steps because the TECAN robotic system was not equipped with on-deck shaker. As discussed above, after removing the sample evaporation and reconstitution steps, most of the sample transfer and SPE steps can be handled automatically by a TECAN robotic handler with a robotic arm. With the addition of an on-deck shaker that has become commercially available, all evaporation-free SPE steps can be handled by a robotic liquid handler – i.e., a fully automated sample preparation method.

### 3.3. LC–MS/MS method for the quantitation of analytes I–VI

The selected reaction monitoring (SRM) transitions used for the quantitation of Analytes I–VI are shown in Table 1. Typical SRM mass chromatograms of blank plasma, blank plasma with only IS and human plasma spiked with the analytes at the concentration of the LLOQ are shown in Fig. 5A–C. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either the analytes or the ISs when control plasma blanks were analyzed.

For Assay 1 (Fig. 5A), analyte I was separated from analyte II with retention times of 2.66 and 1.48 min, respectively. For Assay 2 (Fig. 5B), analyte III had a retention time of 1.57 min with excellent peak shape. For Assay 3 (Fig. 5C), analyte IV was well separated from analytes V and VI with retention times of 2.54, 1.64 and 1.32 min, respectively. All three analytes had excellent peak shapes.

### 3.4. Lower limit of quantitation (LLOQ)

As shown in Fig. 5A–C, for Assay 1, the SRM peak intensities of I and II at their LLOQ concentration levels (2.00 ng/nL) were 7.52E03 and 1.19E04, respectively, which were above the sensitivity that are needed for accurate quantitation. For Assay 2, the MRM peak intensity of III was acceptable at its LLOQ concentration (1.00 ng/mL) at 1380 cps. For Assay 3, the SRM peak intensities of IV, V and VI were sufficiently sensitive at their LLOQ concentration (1.00 ng/mL for each) and were 8086, 2333 and 2073, respectively. As shown in Fig. 5, no significant interfering peaks from the control plasma samples (dog, monkey or human) were found at the retention time and in the ion channel of the analytes or the IS.

### 3.5. Extraction recovery and matrix effects

The assay recovery and matrix effects for these three assays are listed in Table 1. For Assay 1, the recoveries for I and II were 44% and 53%, respectively. The matrix effect was 0.88 for both I and II, while the matrix effects for the internal standards, I′ and II′ were 0.89 and 0.88, respectively. When normalized with the matrix effects of their internal standards, there was no observable matrix effect, since the normalized matrix effects were 0.99 and 1.00. For Assay 2, the recovery for III was 58%. The matrix effects for the III and III′ were 0.97 and 1.03, respectively, which was considered to be insignificant as they are close to 1.00 (the absence of a matrix effect). For Assay 3, the recoveries for IV, V and VI were 55%, 69% and 70%, respectively. The matrix effects were 1.17, 1.09 and 1.22 for IV, V and VI, which were compensated well for by the internal standards, IV′, V′ and VI′ whose matrix effects were 1.19, 1.07 and 1.21, respectively. When normalized with internal standards, there was no observed matrix effect since the normalized matrix effects were close to 1.00 (ranging from 0.98 to 1.02 for three analytes).

As discussed above, evaporation-free SPE resulted in moderate extraction recoveries for the analytes with recoveries of 44–70% for all analytes. The elimination of the sample evaporation did not impact the assay quality in terms of analyte responses and HPLC separation. The normalized matrix effects were close to 1.00 indicating no matrix effects observed for these assays.

### 3.6. Calibration standards and quality control samples

All standard curves for these assays were fitted to a $1/x$ weighted quadratic regression model. For Assay 1, standard curves ranged from 2.00 to 1000 ng/mL for I and II in dog plasma. The mean $r^2$ values were > 0.9991 and 0.9989 for both I and II, respectively. The intra-assay precisions, based on four levels of analytical QCs (low, geometric mean (GM), mid and high), were within 5.0% CV and inter-assay precisions were within 2.8% CV for both analytes. The assay accuracy, expressed as %Dev, was within ±6.8% of the nominal concentration values for both analytes.

For Assay 2, the standard curve ranged from 1.00 to 1000 ng/mL for III in monkey plasma. The mean $r^2$ value was 0.9991 for all three runs. The intra-assay precisions, based on four levels of analytical QCs, were within 4.1% CV and inter-assay precisions were within 3.4% CV for III. The assay accuracy was within ±9.9% of the nominal concentration value.

For Assay 3, the standard curves ranged from 1.00 to 1000 ng/mL for IV, V and VI in human plasma. The mean $r^2$ values of three analytes were better than 0.9991 for all three runs. The intra-assay precisions, based on four levels of analytical QCs, were within 4.4% CV and inter-assay precisions were within 1.7% CV for analytes

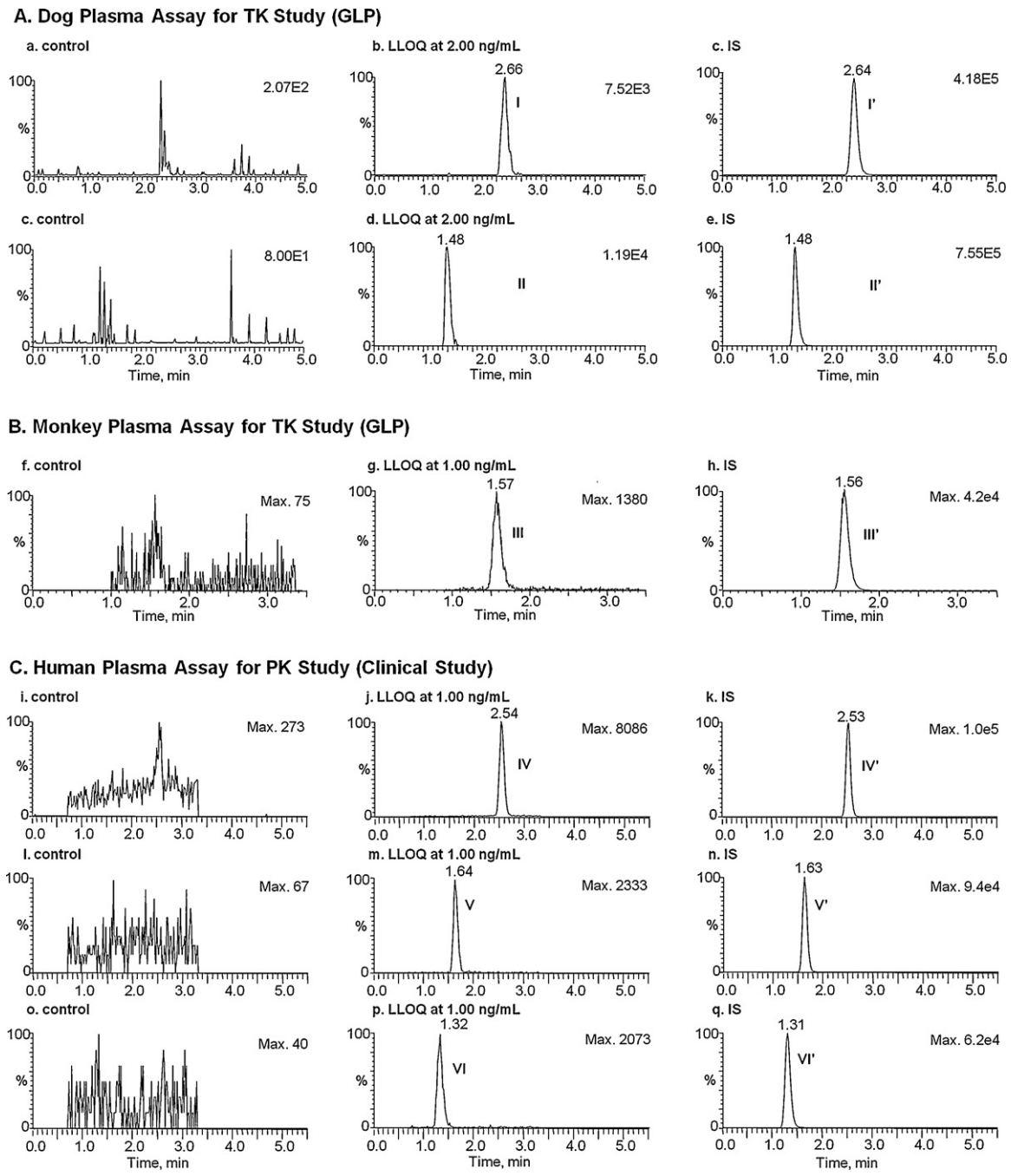


**Fig. 5.** Selected reaction monitoring chromatograms for the analytes I–VI and their internal standards, I′–VI′: (A) dog plasma assay for TK study (GLP); (B) monkey plasma assay for TK study (GLP); (C) human plasma assay for PK study (clinical study); (a, c, f, i, l and o) chromatograms of the analytes obtained from the control plasma matrix; (b, d, g, j, m and p) chromatograms of the analytes obtained from plasma matrix containing the analyte at lower limit of quantitation (LLOQ) and its internal standard; (c, e, h, k, n and q) chromatograms of the internal standards (IS) obtained from the plasma containing only the internal standards.

IV, V and VI. The assay accuracy was within ±9.4% of the nominal concentration values for all three analytes.

In the assays discussed above, except for the SPE plates and elution solvents, which were compound dependent, all SPE conditions such as conditioning, buffer solution and sample elution were very similar, which simplified the method development and TECAN program time significantly. The precision and accuracy of the back-calculated concentrations for the calibration standards are shown in Table 4. The assay precision and accuracy for the analytical QCs are shown in Table 5. The data quality from the three assays presented was excellent, and met the acceptance criteria described in the validation guidance from the FDA [26].

### 3.7. Assay applications

All three LC–ESI/MS/MS methods described in this paper have been applied to the toxicokinetic (TK) studies (Assays 1 and 2) as part of IND enabling studies or to the pharmacokinetic studies in support of clinical studies (Assay 3). Fig. 6A presents the representative TK plasma concentration vs time profiles of BMS-763534 (I) and its metabolite, BMS-790318 (II) in dog plasma following the administration of oral dose of 10 mg/kg/day of BMS-763534 in healthy male dogs with 16.8 ± 6.3 months old and bodyweights of 10.68 ± 0.52 kg ($n$ = 5) using Assay 1. Fig. 6B shows the representative TK plasma concentration vs time profiles of BMS-694153 (III)

**Table 4**
Analytical performance: back-calculated concentrations (ng/mL) for calibration curves for three representative assays.

| Assay 1: nominal concentration (ng/mL) | 2.00 | 4.00 | 10.00 | 25.00 | 50.00 | 100.00 | 250.00 | 500.00 | 750.00 | 1000.00 |
|---|---|---|---|---|---|---|---|---|---|---|
| **BMS-763534 (I)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.90 | 3.89 | 10.23 | 25.55 | 50.72 | 102.99 | 249.97 | 497.19 | 740.75 | 1007.79 |
| %CV | 8.4 | 5.7 | 5.6 | 6.6 | 3.6 | 3.2 | 2.9 | 2.3 | 2.6 | 3.0 |
| %Dev | −5.0 | −2.8 | 2.3 | 2.2 | 1.4 | 3.0 | 0.0 | −0.6 | −1.2 | 0.8 |
| **BMS-790318 (II)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.91 | 3.96 | 10.27 | 25.27 | 50.36 | 101.42 | 251.09 | 498.67 | 741.98 | 1006.07 |
| %CV | 8.9 | 5.6 | 4.6 | 5.6 | 3.6 | 3.9 | 2.6 | 0.9 | 3.7 | 3.7 |
| %Dev | −4.5 | −1.0 | 2.7 | 1.1 | 0.7 | 1.4 | 0.4 | −0.3 | −1.1 | 0.6 |
| Assay 2: nominal concentration (ng/mL) | 1.00 | 2.00 | 5.00 | 10.00 | 50.00 | 100.00 | 250.00 | 500.00 | 750.00 | 1000.00 |
| **BMS-694153 (III)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.02 | 2.02 | 4.99 | 9.86 | 49.71 | 98.17 | 253.05 | 497.44 | 754.58 | 997.29 |
| %CV | 5.9 | 3.0 | 2.6 | 2.9 | 3.8 | 4.8 | 4.9 | 2.9 | 2.7 | 3.0 |
| %Dev | 2.0 | 1.0 | −0.2 | −1.4 | −0.6 | −1.8 | 1.2 | −0.5 | 0.6 | −0.3 |
| Assay 3: nominal concentration (ng/mL) | 1.00 | 2.00 | 5.00 | 10.00 | 50.00 | 100.00 | 250.00 | 500.00 | 750.00 | 1000.00 |
| **BMS-562086 (IV)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.02 | 1.97 | 4.96 | 9.97 | 49.54 | 100.39 | 253.73 | 500.61 | 734.46 | 1014.18 |
| %CV | 3.7 | 4.0 | 2.7 | 2.6 | 1.8 | 1.8 | 2.3 | 3.8 | 3.3 | 5.3 |
| %Dev | 2.0 | −1.2 | −0.8 | −0.3 | −0.9 | 0.4 | 1.5 | 0.1 | −2.1 | 1.4 |
| **BMS-749241 (V)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.03 | 1.97 | 4.93 | 10.02 | 49.54 | 99.53 | 253.80 | 497.83 | 746.28 | 1003.11 |
| %CV | 5.5 | 3.1 | 2.7 | 1.8 | 2.3 | 2.0 | 1.7 | 1.9 | 1.5 | 0.7 |
| %Dev | 3.2 | −1.2 | −1.4 | 0.2 | −0.9 | −0.5 | 1.5 | −0.4 | −0.5 | 0.3 |
| **DPH-123554 (VI)** | | | | | | | | | | |
| Mean ($n$ = 6) | 1.01 | 1.96 | 4.96 | 10.12 | 50.09 | 100.39 | 252.34 | 499.72 | 740.87 | 1006.67 |
| %CV | 3.6 | 3.2 | 3.0 | 1.7 | 2.6 | 1.4 | 1.3 | 2.2 | 1.7 | 2.9 |
| %Dev | 1.0 | −2.3 | −0.8 | 1.2 | 0.2 | 0.4 | 0.9 | −0.1 | −1.2 | 0.7 |

**Table 5**
Assay precision and accuracy for three representative assays ($n$ = 18).

| Assay 1: nominal concentration (ng/mL) | LLOQ (2.00) | Low QC (6.00) | GM QC (50.00)[a] | Mid QC (400.00) | High QC (800.00) | Dil QC (5000.00)[b] |
|---|---|---|---|---|---|---|
| **BMS-763534 (I)** | | | | | | |
| Mean observed concentration (ng/mL) | 1.87 | 5.68 | 49.56 | 400.67 | 803.40 | 5056.12 |
| %Dev | −6.5 | −5.3 | −0.9 | 0.2 | 0.4 | 1.1 |
| Between run precision (%CV) | 9.1 | 2.8 | 1.5 | 1.1 | 2.4 | 4.0 |
| Within run precision (%CV) | 8.1 | 5.0 | 2.8 | 1.9 | 2.8 | 2.6 |
| **BMS-790318 (II)** | | | | | | |
| Mean observed concentration (ng/mL) | 1.86 | 5.59 | 49.65 | 406.61 | 801.80 | 5013.35 |
| %Dev | −7.0 | −6.8 | −0.7 | 1.7 | 0.2 | 0.3 |
| Between run precision (%CV) | 11.2 | 0.0 | 1.9 | 1.5 | 1.9 | 4.7 |
| Within run precision (%CV) | 6.2 | 3.0 | 2.3 | 3.2 | 2.9 | 3.5 |
| Assay 2: nominal concentration (ng/mL) | LLOQ (1.00) | Low QC (3.00) | GM QC (40.00) | Mid QC (400.00) | High QC (800.00) | Dil QC (5000.00) |
| **BMS-694153 (III)** | | | | | | |
| Mean observed concentration | 1.10 | 3.14 | 37.65 | 430.55 | 878.93 | 5253.09 |
| %Dev | 10.0 | 4.7 | −5.9 | 7.6 | 9.9 | 5.1 |
| Between run precision (%CV) | 0.0 | 2.2 | 3.4 | 2.8 | 3.3 | 4.5 |
| Within run precision (%CV) | 7.2 | 4.1 | 2.2 | 2.5 | 2.3 | 1.8 |
| Assay 3: nominal concentration (ng/mL) | LLOQ (1.00) | Low QC (3.00) | GM QC (35.00) | Mid QC (400.00) | High QC (800.00) | Dil QC (5000.00) |
| **BMS-562086 (IV)** | | | | | | |
| Mean observed concentration | 1.01 | 3.10 | 37.37 | 429.12 | 873.89 | 5057.31 |
| %Dev | 1.0 | 3.3 | 6.8 | 7.3 | 9.2 | 1.1 |
| Between run precision (%CV) | 2.5 | 0.0 | 0.6 | 1.4 | 1.0 | 0.0 |
| Within run precision (%CV) | 4.0 | 2.0 | 2.0 | 1.7 | 4.4 | 1.6 |
| **BMS-749241 (V)** | | | | | | |
| Mean observed concentration | 1.00 | 3.00 | 36.11 | 409.95 | 833.68 | 4914.87 |
| %Dev | −0.4 | −0.1 | 3.2 | 2.5 | 4.2 | −1.7 |
| Between run precision (%CV) | 3.8 | 0.1 | 0.8 | 0.0 | 1.7 | 1.2 |
| Within run precision (%CV) | 3.7 | 1.8 | 1.9 | 1.6 | 2.0 | 2.2 |
| **DPH-123554 (VI)** | | | | | | |
| Mean observed concentration | 1.03 | 3.20 | 38.28 | 435.02 | 871.37 | 4776.05 |
| %Dev | 3.6 | 6.6 | 9.4 | 8.8 | 8.9 | −4.5 |
| Between run precision (%CV) | 3.6 | 1.1 | 1.3 | 1.2 | 0.9 | 0.6 |
| Within run precision (%CV) | 3.5 | 1.6 | 1.9 | 1.7 | 2.0 | 1.5 |

[a] One QC sample was eliminated from statistical calculation due to sample preparation error.
[b] Dilution factor = 100.

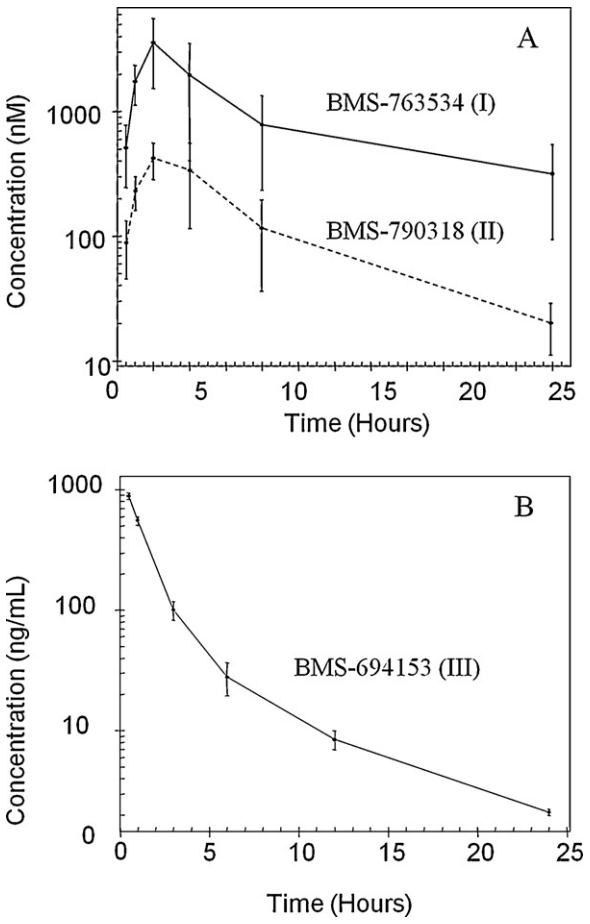


**Fig. 6.** (A) Plasma concentration–time profiles for BMS-763534 (I) and its demethylated metabolite, BMS-790318 (II), in dogs on Day 1 following oral administration of 10 mg/kg/day of BMS-763534 in male dogs ($n = 5$); (B) plasma concentration–time profiles for BMS-694153 (III) in monkey on Day 1 following subcutaneous administration of 2.0 mg/kg/day of BMS-694153 in male monkeys ($n = 3$).

in monkey plasma following the administration of a subcutaneous dose of 2.0 mg/kg/day of BMS-694153 in healthy male monkeys with $35.4 \pm 2.1$ months old and bodyweights of $3.13 \pm 0.57$ kg ($n = 3$) using Assay 2. The LLOQ of each assay is good enough to measure the concentrations of the samples from even the lowest doses. In addition, the same methodology was successfully applied to the simultaneous analysis of I and II in rat EDTA plasma, the quantitation of III in rat EDTA plasma, as well as several other assays where only 150–200 μL of each TK sample was collected. By using 50 μL of the plasma sample for the assay, excellent assay precision and accuracy were also achieved with the BMS-custom tube for direct sample transfer and evaporation-free SPE.

### 3.8. Discussion

In most of the reported automated sample extraction methods, individually manually decapping and recapping a sample tube was still required for regulated bioanalysis due to the use of capped sample storage tubes. For a typical single analytical run with 96 samples in the run, it could take up to 30–45 min to manually decap and recap the sample tubes. However, by using cap-piercing for direct sampling, the time could be saved. In addition, the cap-piercing for direct sampling strategy can be incorporated into any exiting robotic liquid handlers with fixed tips (such as TECAN® or JANUS® systems) without additional capital investment, such as in standalone decapper systems. The newly designed tubes described here allow an accurate aliquot volume of 25–50 μL or more from any sample volume of 100 μL and up to 5 mL in the tubes, with an extreme case of a 50 μL aliquot from a ample volume of 75 μL in the tube. They can be used for majority of regulated bioanalysis for non-clinical and clinical studies.

Similar to any fully automated sampling system, there are potential issues with cap-piercing direct sampling, such as inaccurate sampling due to partially gelled plasma, bubbles in the samples or clogged tips. These issues are not uncommon with any other automated sample preparation methods without using cap-piercing direct sampling, which can be partially resolved by using built-in configuration functions with a robotic liquid handler, such as proper liquid class, liquid level detection and bubble detection functions. To avoid any un-expected interruption of the operation due to bubbles or clogs observed in samples, a brief centrifugation at mid speed is recommended before sample preparation. Optimization of the liquid handling workstation by optimizing the liquid transfer program is also essential to minimize the tip carry-over.

In the assays reported here, all samples that needed to be diluted were manually diluted before placing on the TECAN. Recently, we reported a robotic sample preparation program (RSPP) that was used to automatically dilute and prepare samples using a robotic liquid handler with disposable tips [27]. Similar automated sample dilution with fixed tips was proven to be technically feasible, but was not used in production due to the extra attention needed to determine potential dilution effects caused by the washing solvent [28,29]. To eliminate the dilution effect, pre-wetting or conditioning of the tips are essential for achieving accurate dilution results [28,29].

## 4. Conclusion

Automated cap piercing for direct sample transfer not only saved time and avoided operation error during sample extraction, but also eliminated the concerns with potential spillage of hazardous biofluids collected from patients with infectious diseases. By using evaporation-free solid phase extraction, all steps that required manual intervention have been successfully eliminated by taking into consideration the use of weaker SPE elution solvents followed by dilution with aqueous solution. From the three assays presented, adequate sensitivities were achieved with moderate extraction recoveries. This approach is generic and flexible and can be applied to different types of analytes. These assays exhibited high throughput, yet excellent data quality that met the requirement for submission to regulatory agencies (e.g., the FDA or the European Medicines Agency (EMA)) in support of non-clinical and clinical studies for evaluating the safety and/or efficacy of drug candidates. This methodology was capable of achieving full automation for sample preparation for regulated LC–MS/MS bioanalysis, and has been successfully applied to large TK and PK studies in support of multiple programs in our laboratory.

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