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A fully automated plasma protein precipitation sample preparation method for LC–MS/MS bioanalysis

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Abstract

This report describes the development and validation of a robust robotic system that fully integrates all peripheral devices needed for the automated preparation of plasma samples by protein precipitation. The liquid handling system consisted of a Tecan Freedom EVO® 200 liquid handling platform equipped with an 8-channel liquid handling arm, two robotic plate-handling arms, and two plate shakers. Important additional components integrated into the platform were a robotic temperature-controlled centrifuge, a plate sealer, and a plate seal piercing station. These enabled unattended operation starting from a stock solution of the test compound, a set of test plasma samples and associated reagents. The stock solution of the test compound was used to prepare plasma calibration and quality control samples. Once calibration and quality control samples were prepared, precipitation of plasma proteins was achieved by addition of three volumes of acetonitrile. Integration of the peripheral devices allowed automated sequential completion of the centrifugation, plate sealing, piercing and supernatant transferral steps. The method produced a sealed, injection-ready 96-well plate of plasma extracts. Accuracy and precision of the automated system were satisfactory for the intended use: intra-day and the inter-day precision were excellent (C.V. < 5%), while the intra-day and inter-day accuracies were acceptable (relative error < 8%). The flexibility of the platform was sufficient to accommodate pharmacokinetic studies of different numbers of animals and time points. To the best of our knowledge, this represents the first complete automation of the protein precipitation method for plasma sample analysis.
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Keywords: Fully automated; Plasma; Sample preparation; LC–MS/MS; Bioanalysis

1. Introduction

Rapid and reliable high throughput bioanalysis of drug and metabolite concentrations in plasma samples is essential for pharmacokinetic, pharmacodynamic, and toxicokinetic studies. Liquid chromatography coupled with triple quadrupole mass spectrometers (LC–MS/MS) is the tool of choice for bioanalysis due to its sensitivity, selectivity, specificity, and robustness. In recent years, significant progress has been made in increasing the throughput of LC–MS/MS bioanalysis through various chromatographic approaches such as ballistic gradient elution [1–7] and column switching [8–12]. These improvements, while contributing greatly to the productive support of discovery-stage preclinical pharmacokinetics studies, have shifted the bottleneck to the sample preparation step, which generally requires tedious manual labor.

Three sample preparation methods are commonly employed for quantitative LC–MS/MS analysis of drugs in plasma: protein precipitation with a miscible organic solvent, liquid–solid phase extraction, and liquid–liquid extraction. Many partially automated methods employing robotic liquid handlers have been reported [13–25], mostly for the liquid–solid phase extraction and liquid–liquid extraction techniques, in light of their efficiency in sample cleanup and amenability to automation in the 96-well plate format [20–23]. Although the analyte extraction steps are fully automated, a disadvantage is that manual intervention is still required for steps involving evaporation of the extraction solvent and reconstitution of the dried residue [24,25].

Protein precipitation with miscible organic solvents (usually acetonitrile or methanol) is the most commonly used plasma sample preparation method because of its low cost and minimal method development requirements. The latter feature is

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especially valuable for support of preclinical pharmacokinetic studies conducted during the lead optimization stages of drug discovery, where rapid development of assays for new compounds is essential. This method, involving addition of organic solvent, and centrifugation to separate the resultant protein precipitates from the analyte, provides sufficient clean-up for most LC–MS analyses. Recently reported methods describing the automation of liquid handling steps of the protein precipitation method still require manual plate transfers to accomplish plate sealing, vortexing, centrifugation and plate seal removal [26–28]. One approach [28] uses vacuum filtration for the removal of the precipitated proteins, rather than centrifugation, however manual intervention is still needed for plate sealing. While improved over traditional methods, the requisite manual plate transfers limit the operator's ability to perform additional tasks in parallel.

Described herein is a fully automated protein precipitation-based plasma sample preparation platform. A distinguishing feature is the integration of on-deck plate shakers, centrifuge, plate sealer, and plate seal piercing stations that enables automation of both the liquid handling and plate handling steps. To our knowledge, this represents the first automated protein precipitation method that allows completely unattended operation for preparation of plasma samples that are ready for LC–MS/MS analysis.

2. Experimental

2.1. Materials

HPLC grade acetonitrile, methanol, and water were purchased from Burdick & Jackson. DriSolv dimethyl sulfoxide (EMD) and reagent grade formic acid (Sigma–Aldrich) were obtained from respective vendors. Aluminium pierceable seal was obtained from Velocity 11 (Menlo Park, CA). Abgene deepwell 96-well plates (0.8 mL capacity), 1.2 mL glass-coated 96-well plates, and Tecan 200 μL and 1 mL conductive pipette tips were used for sample preparation.

2.2. Instrumentation

The equipment platform was a Tecan Freedom EVO® 200 liquid handling unit equipped with 8-channel liquid handling arm along with two robotic arms, and two on-deck Te-Shake® plate shakers (Tecan Group Ltd., Durham, NC). Peripheral devices were robotic temperature controlled centrifuge (Hettich Rotanta® 46 RSC, Tecan Group Ltd.), plate sealer (PlateLoc®, Velocity 11) and plate seal piercing station (PlatePierce®, Velocity 11).

2.3. Integration of robotic liquid handling system with peripheral devices

As shown in Fig. 1, an *XY*-axis robotic arm was installed at the left side of the liquid handling arm to facilitate on-deck transfer of 96-well plates to the various locations. A second *Z*-axis robotic arm was configured at the right side of the liquid handling arm to enable movement of 96-well plates in and out of the centrifuge that was bolted to the floor underneath the deck on the far right side. Plate sealer and plate seal piercing stations were located on the left side of the deck. Two on-deck shakers were used for automated vortex mixing of quenched plasma samples. To handle the varied number of samples produced in pharmacokinetic studies of different designs, a custom 12-position plate hotel housed an assortment of balance plates for use in the centrifugation step. The platform was configured to hold six boxes

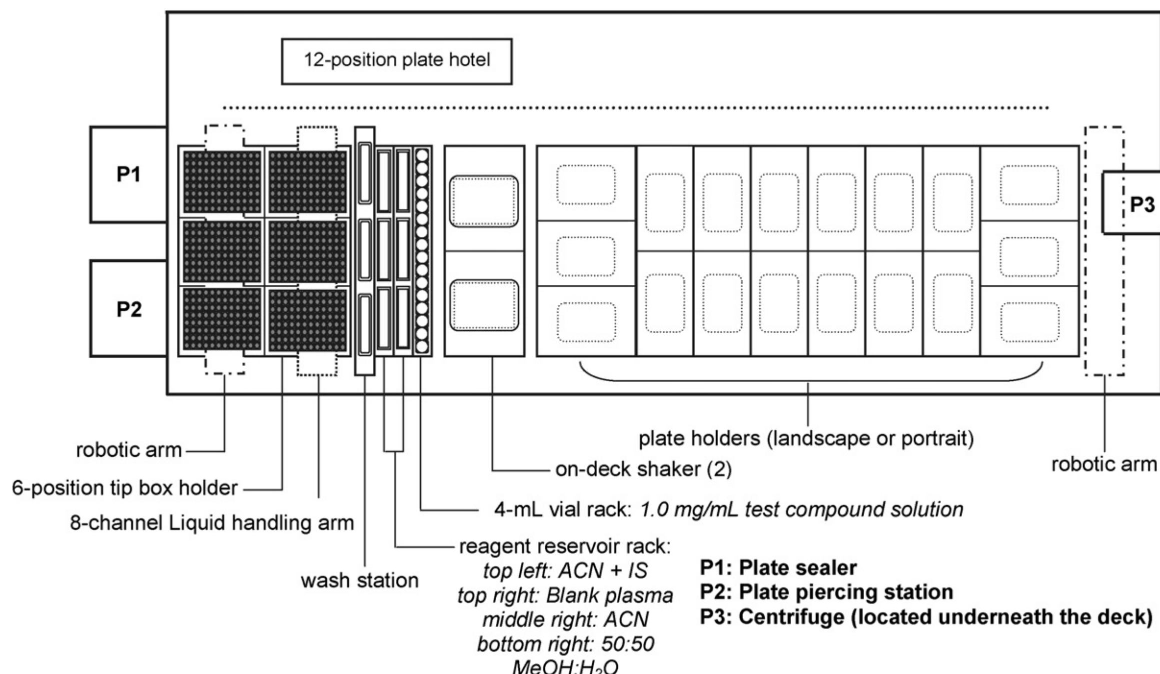

Fig. 1. Schematic diagram of the automated system.

of liquid handling tips including five boxes of 200 μL capacity tips and one box of 1 mL tips (96 per box). To the right side of the wash station were placed two trough holders that could hold up to six different liquid reagents in capacities ranging from 5 to 100 mL. A 16-position tube rack that was installed to the right side of the trough racks held a 4-mL glass vial containing the analyte stock solution. Six portrait- and two landscape-type plate carriers were installed on the deck within the access range of the 8-channel liquid handling arm. Although a standard 96-well plate format was used most commonly, a 24-position block was also included in the system to handle samples received in microcentrifuge tubes or other single tube formats.

In order to ensure reliable unattended plate movement during operation, labware specific calibration profiles were created for both robotic arms for the plate carriers, plate sealer, plate-piercing station, shakers and centrifuge. In addition to volume calibration of liquid handling tips for plasma, also optimized were the liquid handling parameters (aspiration and dispensing rates, air gap volumes, etc.) for DMSO, acetonitrile and 50% methanol in water.

Standard software (EVOware®) was used for the main operating interface. Built-in drivers were used for the on-deck shakers and centrifuge, while custom device drivers required for the control of the plate sealer and plate seal piercing station were provided by Tecan Group Ltd.

2.4. Preparation of calibration standards and quality controls

In one 96-well plate, working solutions, used for the preparation of calibration standard and quality control samples, were prepared by serial dilution of the stock solution (Fig. 2). Starting from a 1.0 mg/mL stock solution of the test compound in DMSO (WS0), twelve working solutions were prepared at 100,000 μg/L (WS1), 25,000 μg/L (WS2), 15,000 μg/L (WS3), 10,000 μg/L (WS4), 5000 μg/L (WS5), 1500 μg/L (WS6), 500 μg/L (WS7), 150 μg/L (WS8), 50 μg/L (WS9), 15 μg/L (WS10), 5 μg/L (WS11) and 1.5 μg/L (WS12) using 50% methanol in water as the diluent. Mixing was achieved by three successive aspirations and dispensings within the sample well. Calibration standards were prepared by transferring 10 μL of each working solution (WS2–WS12) and mixing with 50 μL of blank plasma in the sample preparation plate in final test compound concentration of 0.3–5000 μg/L. Quality control samples were prepared similarly from the same working solution as standard samples (WS10, 9, 7 and 5) in final nominal concentrations of 3, 10, 100 and 1000 μg/L. For the typical early preclinical pharmacokinetic studies, there are two sets of 11 standard samples, and 12 quality control samples. Liquid level sensing was turned on during aspiration of blank plasma from the corresponding reagent reservoir.

2.5. Automated plasma sample preparation

The sample preparation process was organized into ten discrete steps including five liquid handling steps: (1) blank plasma transfer, (2) unknown plasma samples transfer, (3) preparation of calibration standard and QC samples, (4) protein precipitation of plasma samples and (5) supernatant transfer (Fig. 3).

For unknown samples (typically received in a 96-well plate), 50 μL of thawed plasma was transferred into the sample preparation plate. The concentrations of the test compound in the initial timed samples collected after intravenous bolus administration (standard dose was 0.5 mg/kg) frequently exceeded the highest calibration standard. Consequently, to minimize reassays certain samples (drawn at 0.083 and 0.167 h post-dose) were chosen for automatic ten-fold dilution – 5 μL of test plasma was added to 45 μL of blank plasma. To minimize system errors, liquid level sensing was turned off during the sample aspiration step; instead, pipette tips were positioned at a pre-defined position (2 mm above Z-max) within the well to accommodate variations in sample volume. An additional 10 μL of 50% methanol solution was added to each unknown plasma sample in order to compensate for volume differences from the calibration standard samples.

To precipitate plasma proteins, 140 μL of acetonitrile containing an internal standard was added using the multiple dispense mode into each well containing a calibration standard, quality control, or unknown plasma sample. The sample preparation plate was then transferred to the plate sealer by the left robotic arm for placement of an aluminum foil seal. The sealed plate was then moved by the same arm to the on-deck shaker. After vortex mixing for 15 min at 1350 Hz, the plate was transferred back to the previous deck location by the left robotic arm to allow transportation into the centrifuge by the right robotic arm that has limited on-deck range in the *X*–*Y* direction, but can reach into the centrifuge underneath the platform deck. To pellet the denatured proteins, the program then signaled the centrifuge to run for 15 min at 4500 rpm (∼2200g; temperature maintained at 20 °C) with acceleration and deceleration both set at the max-

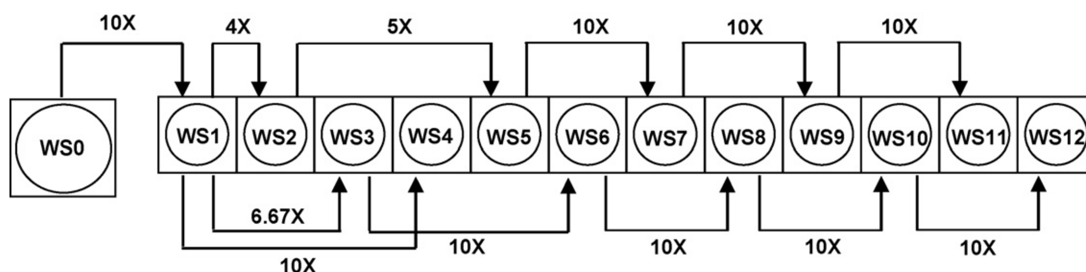

Fig. 2. Dilution scheme for preparation of working solution for calibration standards and QC samples.

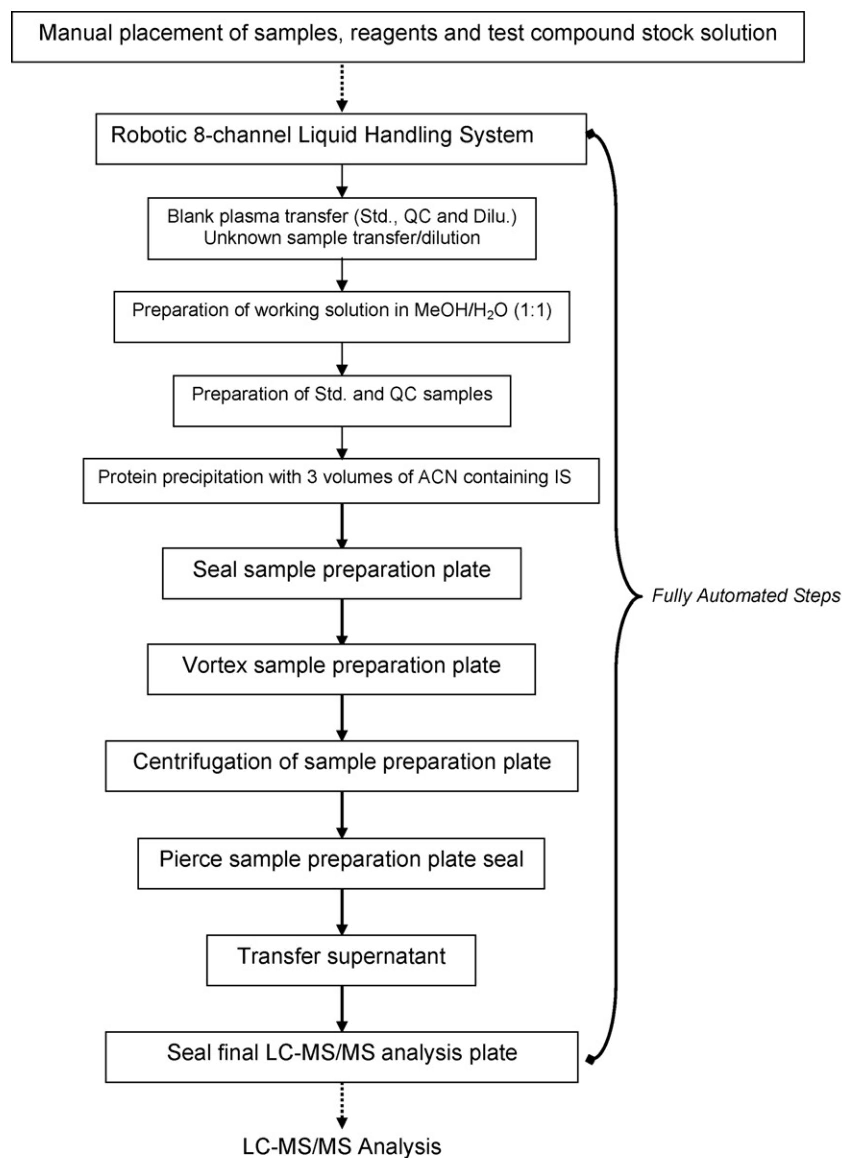

Fig. 3. General work flow for the automated system.

imum rates. After centrifugation, the sample preparation plate was moved by the robotic arms to the plate seal piercing station. Once the seal was pierced, the plate was again moved to the plate shaker, which held the plate securely and enabled the reliable transfer of 120 μL of the plasma supernatants to a fresh plate. This plate containing the supernatants was returned to the plate sealer in the optional final step for placement of an aluminum foil seal. Before LC–MS/MS analysis, the aluminium foil seal was replaced offline with a silicone-rubber mat to minimize solvent evaporation.

2.6. LC–MS/MS conditions

Quantification of Compound **1** (Fig. 4) in plasma was conducted on an API3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Cohesive Aria LX-2, two Agilent 1100 binary pumps (Palo Alto, CA), a CTC HTS PAL autosampler (CTC Analyticas, Switzerland) coupled with one refrigerated (8 °C) cool stack, and a Cohesive VIM module (Franklin, MA). Separation was achieved with Shiseido Capcell PAK UG120 column (3 mm, 2.0 mm × 50 mm, Phenomenex, Torrance, CA) eluted using a binary gradient of 0.1% formic acid aqueous solution (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The initial mobile phase of 5% mobile phase B was held for 0.5 min then increased linearly to 95% of mobile phase B over 0.5 min. The solvent composition was held at 95% of mobile phase B for 2 min, and then returned to the initial condition for 1.5 min. The mobile phase flow rate was set at 600 μL/min with 50% of the flow directed into the mass spectrometer. Sample injection volume was 20 μL.

Positive ionization mode was used on the API3000 mass spectrometer equipped with a turbo ion spray source. Instrument settings were source temperature of 400 °C, nebulizer gas 10 arbitrary units, curtain gas 12 arbitrary units, turbo gas 10 arbitrary units and ion spray voltage at 5000 V. The collision gas in

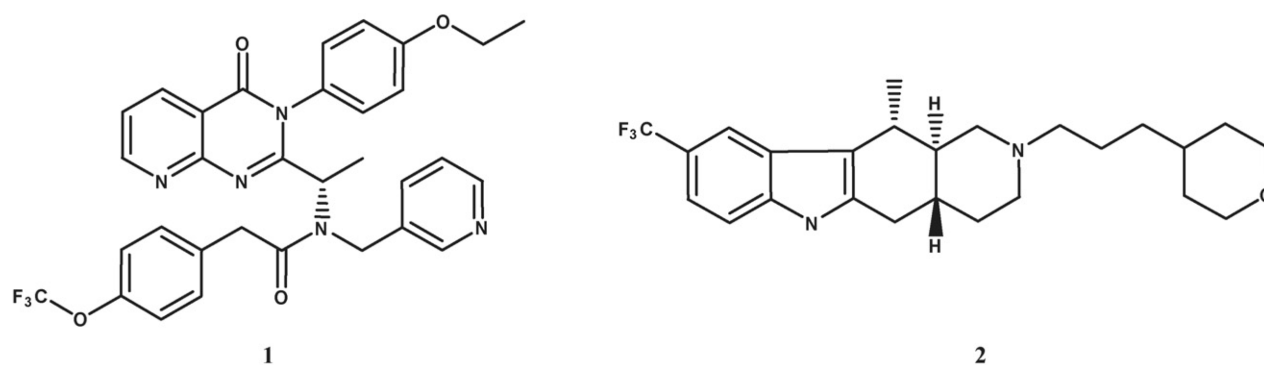

Fig. 4. Molecular structures of Compounds **1** and **2**.

Q2 was set at eight arbitrary units. Selective reaction monitor (SRM) mode was used for quantification of Compound **1** and internal standard Compound **2** (Fig. 4) with the following transitions respectively: 604.20 → 268.30 and 435.20 → 264.30. The dwell time for each transition was set at 150 ms, with the identical entrance potential at 10 V. The declustering potential, focus potential, collision energy and cell exit potential were optimized to 56, 320, 43 and 16 V for Compound **1**, and 80, 260, 57, and 4 V for Compound **2**, respectively.

Analysis of plasma samples from a dog pharmacokinetic study of Compound **3** were conducted on an API2000 triple quadrupole mass spectrometer (Applied Biosystems) coupled with a Shimadzu 10ADvp HPLC system (Columbia, MD) and a CTC HTS PAL autosampler. Chromatographic conditions were similar to those used for analysis of Compound **1**. Turbo Ion spray source settings on the API2000 triple-quadrupole mass spectrometer were: curtain gas setting at 40 arbitrary units, gas 1 and 2 at 50 and 80 arbitrary units, CAD gas at 4 arbitrary unit, ion spray voltage at 5000 V and ion source temperature at 350 °C. The values for focus potential, entrance potential and cell exit potential were set at 350, 10 and 14 V, respectively. The following SRM transitions were used for quantitative analysis of plasma samples using SRM mode: 635.16 → 242.40 for Compound **3** and 435.20 → 264.30 for Compound **2**, which was also used as an internal standard in this study. The declustering potential, collision cell entrance potential and collision energy were optimized as 46, 24.4, and 47 V for Compound **3** and 80, 19.4 and 57 volts for internal standard.

Analyst 1.4.1 (Applied Biosystems) was used for peak integration and linear regression.

2.7. Accuracy and precision of the fully automated plasma sample preparation platform

Accuracy and precision of the system were evaluated using a set of plasma samples spiked with Compound **1** in final concentrations of 10, 100 and 1000 μg/L. These samples were prepared offline from a separately weighed powder and diluted using volumetric labware. Intra-day performance was assessed by replicate analysis (n = 4–6) of these samples. The accuracy of the dilution step was assessed by ten-fold dilution of the 1000 μg/L samples. Inter-day performance was evaluated by replicate analysis (n = 12–18) over 3 days of analysis. The coefficient of variation and relative error were calculated as previously described [29].

2.8. Intravenous administration of Compound 3 to Beagle Dogs

In an example application of the method, a 7-day pharmacokinetic study in beagle dogs (n = 4) was carried out using Compound **3**, an analogue of Compound **1**. The test article was administered on days 1 and 7 by intravenous bolus injection. Blood samples were collected from each animal by venipuncture pre-dose and 0.0833, 0.167, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h post-dose on days 1 and 7. Blood samples were collected into tubes pretreated with EDTA and kept on wet ice until centrifugation at 1500 × g for 10 min (2–8 °C). Following centrifugation, the resultant plasma was transferred into duplicate tubes and stored frozen at −70 °C prior to LC–MS/MS analysis. Plasma samples of Compound **3** from the study were received in individual tubes, and a pair of special adapters was used to arrange the thawed samples in a format compatible with the orientation of the liquid handling arm. Two final LC–MS/MS analysis plates were generated which contained all unknown samples, two sets of calibration standards, quality control and blank samples (Fig. 5).

3. Results

3.1. Optimization of liquid handling parameters

DMSO was chosen as the default solvent for the initial stock solution due to its ability to dissolve compounds of divergent physicochemical properties. While water is the most easily handled liquid type for a robotic handling system, the limited aqueous solubility of many early drug candidates makes it unsuited as a general solvent for preparation of concentrated working solutions. In contrast, organic solvents (methanol and acetonitrile) are capable of dissolving lipophilic compounds, but present a challenge for automated pipetting with high degrees of precision and accuracy. During solvent optimization, it was found that a binary mixture of water and methanol (1:1) provided the best combination of overall desired properties, and therefore was chosen as the standard solvent for serial dilutions of the calibration standard and QC working solutions. Liquid

	1	2	3	4	5	6	7	8	9	10	11	12	
Standard 1	1	2	3	4	5	6	7	8	9	10	11	B	A
Standard 2	1	2	3	4	5	6	7	8	9	10	11	B	B
QC	1	2	3	4	1	2	3	4	1	2	3	4	C
Double Blank	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	D
Double Blank	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	DB	E
													F
													G
													H

a: LC-MS analysis plate 1

	1	2	3	4	5	6	7	8	9	10	11	12	
Subject A	1	2	3	4	5	6	7	8	9	10	11	12	A
Subject B	1	2	3	4	5	6	7	8	9	10	11	12	B
Subject C	1	2	3	4	5	6	7	8	9	10	11	12	C
Subject D	1	2	3	4	5	6	7	8	9	10	11	12	D
10X Dilution	A2	A3	B2	B3	C2	C3	D2	D3					E
													F
													G
													H

b: LC-MS analysis plate 2

Fig. 5. LC–MS/MS analysis plate layouts. LC–MS analysis plate 1: calibration standard samples (0.3–5000 μg/L, A1–A11, B1–B11): QC samples (3, 10, 100 and 1000 μg/L, C1–C4, C5–C8, C9–C12). Blank samples (B, A12, B12) were blank plasma samples with IS, double blank samples (DB, D1–D12, E1–E12) contained blank plasma only. LC–MS analysis plate 2: unknown plasma samples of subjects A–D (pre-dose and 11 post-dose time points; A1–A12, B1–B12, C1–C12 and D1–D12, respectively.). Dilution samples of two early time points (0.0833 and 0.167 h post-dose) from each subject (E1–E8).

handling parameters, such as aspiration and dispensing speed, air gap volumes before and after aspiration, and pipetting volume calibration, were optimized for handling of plasma and acetonitrile.

Carryover is a common problem that hampers assay precision and accuracy during sample transfer and dilution steps. Even with disposable pipette tips, it still was a challenge for viscous liquids, such as plasma and DMSO because these fluids occasionally back-splash onto the tip cone of each pipetting channel during the aspiration step. Consequently, washing steps were added after transferring of these liquids; tip cones were flushed with 5 mL of water.

Despite the use of anticoagulants, clots often occur in plasma samples from pharmacokinetic studies, and these can present an obstacle to accurate automated pipetting. In the author's laboratory, EDTA is used as the standard anticoagulant for all pharmacokinetic studies to minimize clot formation [30]. Unknown samples were visually inspected for clots prior to loading on the platform deck and if visible clots were present, a brief 15–30 s off-line centrifugation pelleted the clots and prevented the clogging of pipet tips during automated liquid transfer.

Liquid level sensing was turned on during aspiration of liquid reagents from trough reservoirs to avoid unexpected low liquid level errors that could lead to failure of an operation. With the sensors turned on, it was also possible to use smaller reagent volumes without compromising system performance. However, the liquid level sensing was turned off during serial dilution and transfer of unknown plasma samples due to limitations of current sensing technology. During the serial dilution process, the sensor was not robust enough to distinguish the real liquid level from interference by tiny air bubbles generated during mixing steps. Instead, fixing the tip position during aspiration at 2 mm above a predefined maximum level (Z-max) and optimizing aspiration speed and air gap settings allowed accurate and precise dilutions without liquid level sensing. In pharmacokinetic study samples, variable sample volumes, fine particulates and tiny air bubbles on the surface of the plasma presented a challenge to the use of liquid level sensing during transfer without triggering errors. This was resolved by fixing the tip position during aspiration at 1.5 mm above Z-max level of the 96-well plate, optimizing aspiration speed and pre-aspiration tip rinses. Positioning the tip thusly also minimized the risk of clogging by thrombin clots that were floating on the surface.

3.2. *Optimization of plate manipulations*

In order to provide maximal efficiency, a primary objective was to complete the sequential plate manipulations without human intervention. Integration of a plate sealer minimized solvent evaporation and spillage, while incorporation of a plate seal piercing station on the deck eliminated the need for manual removal of the plate seal. Selection of both integrated peripheral devices over several available options was based on assessment of their system performance, robustness and readiness of integration. Test results demonstrated that automatic vortex mixing of quenched plasma samples using the on-deck shakers provided analyte extraction efficiency comparable to standard offline mixing. The dedicated *Z*-axis robotic arm was necessary for automated movement of 96-well plates in and out of the centrifuge. Selection of the centrifuge was based on evaluation of features such as G-force, temperature control and space requirements. Higher G-force generally resulted in cleaner supernatants and shorter run time, which was an important consideration for efficiency. Holding samples at room temperature during centrifugation minimized generation of moisture that could lead to the failure of downstream plate manipulations and protected the integrity of test compounds. Occasionally, a malfunction occurred during supernatant transfer that was caused by pipette tips becoming entangled in the pierced aluminum foil seal. This was eliminated by moving the sample preparation plate during final supernatant transfer to an on-deck shaker, which contained a gripping mechanism that securely held the plate. A temporary seal applied to the LC–MS/MS analysis plate was set up as an optional last step to minimize any solvent evaporation and spillage before the application of a silicone-rubber mat for LC–MS/MS injection.

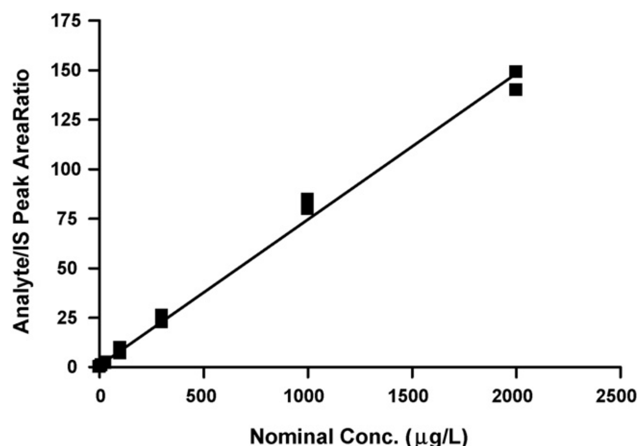

Fig. 6. Representative regression curve of the validation study.

3.3. Accuracy and precision of the automation platform

System accuracy and precision of the automated sample preparation method was evaluated by analysis of rat plasma spiked with varying concentrations of Compound **1**. Best calibration results were obtained by linear regression of peak area ratio vs. concentration with $1/X^2$ weighting; R-squared values for calibration lines exceeded 0.99 (Fig. 6).

Relative errors of intra-day results ranged from 1.70 to 7.20% at the three tested concentrations, whereas coefficient of variation was between 1.58 and 4.80% (Table 1). Inter-day performance, assessed via replicate analysis ($n = 18$ per concentration) over a 3-day period, showed that the relative error over the 10–1000 μg/L range was less than 5.31%, and the coefficient of variation was less than 4.17% (Table 1). The accuracy and precision was maintained in the 1000 μg/L samples that underwent ten-fold dilution prior to extraction (Table 1). These test results indicated that the robotic method provided accuracy and precision that was more than sufficient for the intended use.

Table 1
Accuracy and precision of the analysis of Compound **1** plasma samples using the automated plasma sample preparation system

Nominal concentration (μg/L)	*n*	Average (μg/L)	Relative error (%)	Coefficient of variation (%)
Day 1				
10	6	10.4	3.80	2.79
100	6	102	1.70	4.17
1000	6	969	−3.10	3.73
1000[a]	4	945	−5.53	2.17
Day 2				
10	6	10.3	2.80	1.97
100	6	107	6.60	3.29
1000	6	946	−5.36	3.90
1000[a]	4	962	−3.85	1.58
Day 3				
10	6	10.5	5.06	4.13
100	6	107	7.20	4.80
1000	6	934	−6.64	3.24
1000[a]	4	968	−3.20	3.91
Inter-day				
10	6	10.4	3.68	3.12
100	6	105	5.31	4.17
1000	6	949	−5.11	3.56
1000[a]	4	958	−4.19	2.68

[a] 10× dilution of 1000 μg/L samples.

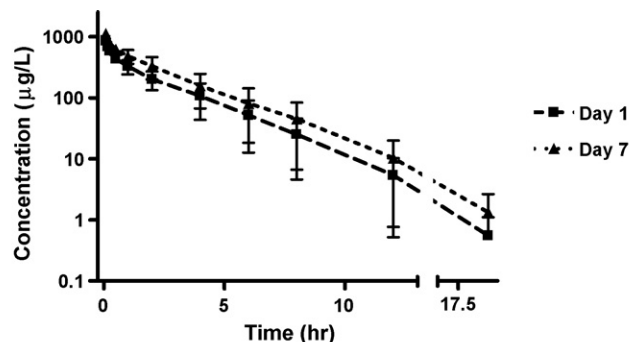

Fig. 7. Plasma concentration-time profile in dogs on days 1 and 7 following i.v. administration of 0.5 mg/kg of Compound **3** on days 1 and 7.

3.4. Application of the automated method in pharmacokinetics studies

The robotic sample preparation system was applied in a 7-day intravenous dose beagle dog pharmacokinetic study of Compound **3** at 0.5 mg/kg. No significant endogenous interference was encountered in this analysis, indicating that the automated sample preparation method provided acceptable sample cleanup. The LOQ of 0.3 μg/L, along with satisfactory linearity over four orders of magnitude (0.3–5000 μg/L) allowed characterization of the concentration–time profile of Compound **3** over eight half-lives (Fig. 7).

3.5. Discussion

System reliability was a key consideration in designing the plasma sample preparation system. Selection of mature technologies and modular workflow programming provided a robust and flexible platform for support of early-stage bioanalysis. With over 1 year into production supporting drug discovery projects, the system has demonstrated excellent flexibility in accommodating over 80% of all bioanalytical samples in the author's laboratory. The inability to precisely and accurately handle small sample volumes (<50 μL) and different biological matrices has limited application of the system to the other studies. In the support of discovery bioanalysis, reassay because of errors or malfunctions during sample preparation, LC–MS instrument operation, etc. has accounted for less than 5% of total runs. Indeed, implementation of the automated system resulted in fewer run rejections attributable to errors in sample preparation than the previous manual procedure. Hardware malfunctions accounted for <1% of runs.

Scheduled preventive maintenance and unscheduled hardware repair have accounted for <10% of available instrument time. Routine maintenance includes a daily flush of the system's liquid tubing system and verification of tip cone tightness.

A monthly tubing system cleaning with 6% bleach, calibration check of both robotic arms and system precision and accuracy tests also have assured quality performance.

Under the configuration of one study per run, the automated system has the capacity of handling up to six typical pharmacokinetic studies per day (up to 48 plasma samples per study), with typical run time of 75 min, which is the approximate time per run available to operators for other duties. Recently, the daily capacity of the system was nearly doubled by further program optimization that allowed in one run the preparation of two typical pharmacokinetic studies. Modifications included putting two sets of working solutions in one plate and using the second sample plate to balance the rotor during centrifugation. Ongoing efforts are aimed at optimizing the deck space usage to increase further the number of studies per run, and utilizing scheduling features for maximum flexibility. Automating electronic input of sample tracking information and data transfer between the LIMS system (i.e. WATSON), liquid handling software, and LC–MS operational software would allow greater efficiencies. Additional efforts are directed at automating procedures for other biological matrices, including brain tissue homogenates to facilitate timely assessment of brain distribution of CNS drug candidates.

In the author's laboratory to support discovery projects, the high organic content (∼75% acetonitrile) of the final supernatant generally was not an obstacle to reasonable chromatography. On rare occasions, changing the column and drying down and reconstituting with appropriate solvent was required to analyze polar analytes. Addition of water or aqueous buffer to the final supernatant could be incorporated into the program to achieve acceptable chromatographic performance.

4. Conclusion

A fully automated protein precipitation-based plasma sample preparation system using a commercially available liquid handler platform was developed for robust bioanalytical support of early preclinical pharmacokinetic studies. In addition to automatic liquid handling, unattended operation was achieved by integrating plate sealer, plate seal piercing station, centrifuge, and shakers. To our knowledge, this represents the first report of a system that allows completely unattended processing from the starting point of thawed plasma samples to the final LC–MS/MS injection-ready analysis plate of plasma extracts. The flexible platform provided significant labor savings, ergonomic-friendly operation, and excellent accuracy and precision.

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